The pituitary-gonad relationship in Xenopus laevis.

by

B.G. Townsend

Thesis presented for the degree of Doctor of Philosophy of the University of Edinburgh.

1968

I should like to thank Dr. B.M. Hobson for the help and advice he has given during his very patient supervision of this investigation, and Professor R.J. Kellar, C.B.E., for the interest he has shown in this work.

TABLE OF CONTENTS.

SUMMARY

The suitability of Tricaine methane sulphonate, urethane and ether as anaesthetics for Xenopus laevis was investigated. Tricaine was found to be the best of these anaesthetics and a suitable dose/body weight relationship was established.

Frequency of feeding was shown to have no effect on spermiation and the secondary sex characters of male Xenopus over a period of months.

The proximity of other animals of the same or a different species was shown to inhibit spermiation and the secondary sex characters of male X. laevis. The evidence suggests this effect is due to inhibition of adenohypophyseal gonadotrophin secretion.

Nuptial pads (gloving) in male Xenopus was shown to be controlled by testicular and adrenocortical hormones which are secreted under adenohypophyseal stimulation.

Evidence was obtained which suggests that there are two pituitary gonadotrophins in Xenopus; one which stimulates gametogenesis and the other which stimulates the secretion of a gonadal steroid.

A inhibiting effect of the gonads on the secretion of adenohypophyseal gonadotrophin has been found.

A well controlled technique for investigating in-vitro ovulation of excised Xenopus ovaries has been developed. Ovaries have been shown to ovulate in-vitro

in response to a number of steroids as well as to luteinizing gonadotrophins and adrenocorticotrophic hormone (A.C.T.H.). This is the first report of in-vitro ovulation of excised Amphibian ovaries in response to oestrogens. It is postulated that ovulation in Xenopus is caused by an ovarian steroid secreted as a result of gonadotrophic stimulation.

In-vivo ovulation has been shown to result from injection of adrenocorticosteroids, methyl testosterone and projesterone but not in response to injected oestradiol or A.C.T.H. Hydrocortisone has been found to inhibit the ovulatory response of female X. laevis to a subsequent injection of human chorionic gonadotrophin (H.C.G.).

Cloacal hyperaemia in female Xenopus has been shown to be controlled by an ovarian hormone secreted in response to stimulation by an adenohypophyseal hormone. Ovulation is only stimulated by a gonadotrophin with luteinizing properties but a gonadotrophin with follicle-stimulating activity is more potent in stimulating cloacal hyperaemia.

Gloving, which is essentially a male secondary sex character, was found to be present in some female X. laevis. This gloving is not under the control of the ovaries. Evidence is provided which suggests that it is developed under adrenocortical stimulation.

INTRODUCTION

The results of investigations carried out on various species of Amphibia indicate that these vertebrates have an endocrine system similar to that of the Mammalia. The mechanisms which control the pituitary-gonad relationship in mammals are well established, it is less well defined in the poikilothermic vertebrates.

In this thesis the relationship between the gonads and the adenohypophysis of the South African Clawed Toad, Xenopus laevis is investigated. Two main lines of research were pursued:-

1. The hormonal control by the adenohypophysis of the functioning of the gonads in regulating the release of the gametes and in controlling the development and maintenance of the secondary sex characters.

2. The regulation of the gonadotrophic function of the adenohypophysis of the gonads.

Before presenting the results of my investigations the relevant literature will be reviewed.

Review of Literature.

1. Male Amphibia.

The effect of hypophysectomy on the testis.

In Xenopus laevis, hypophysectomy causes testicular atrophy (Hogben, 1930; Shapiro, 1943; Bellerby and Hogben, 1938; Gitlin, 1947). Bellerby and Hogben (1938)

showed that removal of only the pars distalis is sufficient to cause regression of the testes, and that this is not indirectly due to the reduced rate of metabolism which follows adenohypophysectomy. A similar involution of the testes after adenohypophysectomy has been reported in Bufo arenarum (Giusti and Houssay, 1923, 1924; Houssay and Lascano-Gonzalez, 1929; Houssay, 1949, 1954; Burgos, 1949, 1950a,b; Penhos and Cardenza, 1952), in Rana pipiens (Burgos, 1955; Burgos and Ladman, 1957) and in Rana temporaria and Rana esculenta (van Oordt and van Oordt, 1955; van Oordt, 1956, 1960). In the Urodeles, also, testicular atrophy follows removal of the pituitary (Dodd, 1960).

The effect of hypophysectomy on spermatogenesis.

In R. esculenta, the histological appearance of the testis tubules of animals hypophysectomised during the hibernation period is similar to that of normal animals until the following breeding season when spermatogenesis is initiated in unoperated control animals (Sluiter, van Oordt and Mighorst, 1950). In R. temporaria, the immediate effect of hypophysectomy depends on the time of operation, but spermatogenesis never proceeds in the absence of the adenohypophysis (Sluiter, van Oordt and Grasveld, 1950; van Oordt, Sluiter and van Oordt, 1951, 1952; Lofts, 1961). van Oordt (1956) has stated that, even during hibernation, spermatogenesis may be affected

by hypophysectomy, and this has been confirmed in R. pipiens by Burgos (1955) who found definite histochemical changes in the testes of animals hypophysectomised during hibernation. In B. arenarum spermatogenesis eventually ceases following removal of the pars distalis (Burgos 1950b).

The effect of exogenous gonadotrophins on spermatogenesis.

Spermatogenesis can be stimulated in both hypophysectomised and normal B. arenarum by homoimplantation of pars distalis or repeated injections of extracts of toad pars distalis (A.L.P.) (Houssay and Lascano-Gonzalez, 1929). van Oordt and his colleagues have found that spermatogenesis can be stimulated in both normal and hypophysectomised R. temporaria by injecting frog pituitaries or pregnant mares' serum (P.M.S.) (Sluiter, van Oordt and Grasveld, 1950; van Oordt, Sluiter and van Oordt, 1951, 1952; van Oordt and van Oordt, 1955). A similar situation in B. arenarum is reported by Houssay (1954). In this species, injection of human chorionic gonadotrophin (H.C.G.) is also reported to stimulate spermatogenesis to a greater extent than injection of P.M.S. The latter has a greater stimulatory effect on the interstitial cells (Burgos and Ruffino, 1953). The latter authors found that simultaneous administration of H.C.G. and P.M.S. mimicked the effect of injection of toad pars distalis which stimulates both spermatogenesis and the interstitial cells.

It is probable that separate gonadotrophins similar to follicle-stimulating hormone (F.S.H.) and luteinizing hormone (L.H.) are present in the Amphibia as in higher vertebrates. Evidence that Amphibian testes are regulated by two gonadotrophins is provided by the finding that injection of F.S.H. stimulates principally spermatogenesis in the testes of hypophysectomised R. temporaria whereas L.H. stimulates an increased activity of the interstitial cells and causes spermiation (Lofts, 1961). The term spermiation has been coined to define the release of sperm into the lumen of the testis tubules and their passage into the urine (van Oordt and Klomp, 1946). It has been suggested that F.S.H. stimulates the Sertoli cells to secrete a hormone which regulates spermatogenesis and that the lipid droplets which appear in these cells in hypophysectomised R. temporaria contain a precursor of this hormone. Injection of hypophysectomised frogs with F.S.H. is reported to cause the disappearance of these lipid droplets (Lofts, 1961, 1964).

Stimulation of Spermiation.

The spermiation response to exogenous gonadotrophins varies among the Anura; the majority of species appears only to spermiate in response to gonadotrophin with luteinizing activity. Although Bufo melanosticus and B. arenarum have been reported to spermiate in response to injections of L.H. and F.S.H. (Bhaduri, 1951; Houssay,

1954), Thorburg and Hansen (1951) found that Bufo bufo do not spermiate in response to high doses of F.S.H.

The Ranidae are similar in only spermiating in response to exogenous gonadotrophin with luteinizing activity (Atz and Pickford, 1954; Burgos and Ladman, 1955, 1957; Witschi and Chang, 1958).

The spermiation response of X. laevis to injections of mammalian gonadotrophins is different from that of other Anura. The first quantitative comparison of their spermiation response to different gonadotrophins is that of Hobson and Landgrebe (1954). Using rigidly controlled experimental conditions (Hobson, 1952), they measured the M.E.D. 50 for spermiation of several mammalian gonadotrophins and found that male X. laevis are more sensitive to a gonadotrophin with mainly follicle stimulating activity; equivalent doses of P.M.S. and H.C.G. being 3·3 I.U. and 15·0 I.U. respectively.

A third pituitary hormone, Prolactin, may be gonadotrophic in some vertebrates (Hoar, 1966) but there is little evidence of it having any gonadotrophic activity in Amphibia. Greenblatt, Clark and West (1949) reported that its injection stimulates spermiation in R. pipiens but later reports contradict this (Atz and Pickford, 1954; Burgos and Ladman, 1957) and Houssay, Penhos and Burgos (1953) found it ineffective in stimulating spermiation in B. arenarum.

It is possible that gonadotrophin stimulates spermiation in Anura through the mediation of another hormone. Injection of macerated adrenal tissue stimulates spermiation in R. pipiens (Robbins and Parker, 1952a,b) and injection of adrenalin, isopropyl nor-adrenalin, methyl testosterone, ethinyl oestradiol and deoxycorticosterone acetate (D.O.C.A.) stimulate spermiation in X. laevis (Hobson, 1952, 1954). Injection of adrenalin has also been shown to cause spermiation in Rana spp. but the adrenal glands are not necessary for the spermiation response to injected H.C.G. in R. pipiens, Hyla arborea or Bufo viridens (Sulman, 1951). Injection of adrenalin causes spermiation in several species of toads by causing a fluid uptake by the testes followed by vacuolisation and apical rupture of the Sertoli cells (Houssay, 1954). A similar reaction to adrenalin occurs in R. temporaria (van Oordt, Beenakkers, van Oordt and Stadhouders, 1954) and in this species immersion of the isolated testis in hypotonic saline solution causes a similar fluid uptake and spermiation. It seems certain that changes in the Sertoli cells are involved in spermiation from the cyclical changes which occur in these cells in normal R. esculenta and during the spermiation response to injections in hypophysectomised R. temporaria (Lofts, 1961, 1964).

In R. temporaria, spermiation is believed to occur as a result of the distension of the testicular tubules which

follows an uptake of water by the testes. This occurs after injection of frog pituitary extract and is thought to be the result of depolymerisation of an acid mucopolysaccharide which causes an increase of the colloid osmotic pressure of the testis. Fluid is drawn into the testicular tubules, and its movement along them loosens the sperm bundles from the Sertoli cells and carries them toward the efferent ducts (van Dongen and de Kort, 1959; van Dongen, Ballieux, Geursen and Offermans, 1960). van Oordt (1962) suggests that the mucopolysaccharide is hyaluronic acid and that the pituitary regulates its breakdown by hyaluronidase.

The possibility that a testicular substance is involved in the process of spermiation is supported by the observation that injection of an Anuran testicular extract causes spermiation in R. esculenta. However, similar extracts do not cause spermiation in B. bufo (B. vulgaris), Bufo kisuroensis or R. esculenta (Wille, 1956).

The influence of the testes on male secondary sex characters.

Loss of breeding behaviour following castration has been reported in many species of Anura (Schrader, 1887; Nussbaum, 1905; Steinach, 1910; Baglioni, 1911; Edinger, 1913; Aron, 1926; Christensen, 1931). Baglioni (1911) concluded that an internal secretion of the testis affects nervous centres responsible for amplexus.

The disappearance of thumb pads and male sexual colouring has also been reported in several species. The reappearance of male secondary sex characters has been found to occur in castrated male Anurans implanted with testes (Bresca, 1910; Takahashi, 1923; Welti, 1928; Harms, 1926; Moskowska, 1932; Kinoshita, 1933; Kehl, 1944).

Testicular implants have also been shown to cause the development of male secondary sex characters in ovariectomised female salamanders and toads (Ponse, 1923; Welti, 1925; Noble and Davis, 1928; Noble, 1929).

It is well known that in some species of Amphibia the male secondary sex characters only develop during the breeding season. Seasonal development of the thumb pads has been correlated with changes in the histological appearance of the testicular interstitial cells in Rana spp. (Aron, 1924, 1926; Smith, 1938; Glass and Rugh, 1944; Iwasawa and Asai, 1959). However, not all authors agree that the interstitial cells are responsible for the development and maintenance of the male secondary sex characters in the Amphibia. It has been suggested that the Sertoli cells are responsible for the development of male characters in some Urodeles (Humphrey, 1925; Adams, 1940). Lofts (1964) has suggested that confusion may have arisen because estimates of interstitial cell activity have often been based on the size of cells and their nuclei and the volume of tissue. These are not always

good criteria of a tissue's functional activity, as is indicated by the finding of little change in the size of the nuclei of interstitial cells after hypophysectomy in R. esculenta (Sluiter, van Oordt and Mighorst, 1950). However, cyclic cytochemical changes have been reported in the interstial cells of R. temporaria, and a close correlation has been found between the development of the thumb pads and the build up of lipid droplets in the interstitial cells of R. esculenta (Lofts and Boswell, 1960; Lofts, 1964).

Injection of testosterone has been found to cause development of thumb pad in castrated R. pipiens and B. arenarum (Wolf, 1939; Burgos, 1950, 1951). However, the latter author reported that the clasping reflex is only restored by the joint administration of testosterone and Bufo A.L.P. Berk (1939) was unsuccessful in stimulating development of gloves in adult X. laevis by injecting testosterone propionate.

In larval Anura, the developing gonads are sensitive to androgen treatment. Maintaining tadpoles in dilute aqueous solutions of testosterone propionate has been found to change genetically female animals into males with testes (Gallien, 1937, 1938, 1944, 1955; Mintz and Witschi, 1946; Mintz, 1948). In juvenile B. fowleri, thumb pad pigment has appeared in both males and females after injection of testosterone propionate (Blair, 1946).

Not all the effects of testosterone are stimulatory. In adult Rana spp., administration of testosterone has been found to completely block spermatogenesis (van Oordt and Basu, 1960; van Oordt and Schouten, 1961; van Oordt, 1962). This could indicate an inhibition of endogenous gonadotrophin secretion from the pars distalis.

So far, it has been shown that the Amphibian testis has 2 functions; production of sperm and secretion of an androgenic hormone. Both spermatogenesis and androgen secretion are under adenohypophyseal control, probably through separate gonadotrophins - F.S.H. which stimulates spermatogenesis, and Interstitial Cell Stimulating Hormone (I.C.S.H.) which stimulates androgen secretion. The latter is thought to be identical with L.H. and will be referred to as L.H.

The gonadotrophic function of the pituitary was first discovered in the Mammal by Zondeck and Aschheim (1927) and Smith (1927) working independently. It was later realised that the secretion of gonadotrophins is influenced by the gonads. It was in this way that Moore and Price (1932) explained the hypertrophy which occurs in the remaining gonad after unilateral gonadectomy. It is now generally accepted that there is a "negative feed-back" mechanism. It is also well established that the hypothalamus controls secretion by the adenohypophysis through polypeptide releasing factors liberated into the hypothalamico-hypophyseal portal system. There are believed

to be separate L.H. and F.S.H. releasing factors (L.H.-R.F. and F.S.H.-R.F.). It is now generally accepted that negative feed-back action exercised on the pars distalis by the hormonal secretions of the gonads may work directly on the pituitary but more probably works indirectly via the hypothalamus, (van Oordt, 1962).

Pituitary control of the androgenic function of the testes.

Hypophysectomy has been shown to cause regression of the male secondary sex characters in the toad (Houssay and Giusti, 1930), in R. temporaria (Gallien, 1940), and in R. esculenta (Sluiter et al., 1950). In the intact male toad, it has been shown that daily implantation of toad pars distalis will provoke the appearance of spontaneous or reflex sexual embrace in normal toads (Houssay and Giusti, 1929) and also in hypophysectomised animals (Houssay and Giusti, 1930). That this action of implanted pars distalis is mediated via the testes is demonstrated by its lack of activity in castrated male toads (Houssay and Giusti, 1929), and in castrated male or in intact female Xenopus laevis (Berk, 1939). This is confirmed by the report that, although injections of testosterone cause the development of thumb pads in both male and female B. fowleri, injections of pituitary have this effect only on male toads; i.e. when a testis is present (Blair, 1946). This result is at variance with that of Moszkowska (1932) who found that pituitary

stimulation of castrated male B. (Bombinator) bombina causes the development of thumb pads. Although this finding has been cited as evidence of a possible direct action of the pituitary on the secondary sexual characters in some amphibia (Lofts, 1964), it is also possible that pituitary hormones stimulate the adrenal cortical tissue to secrete an androgen.

Although hypophysectomy has been reported to cause little change in the size of the nuclei of testicular interstitial cells in R. esculenta (Sluiter et al., 1950), the injection of pituitary extracts has been found to cause an increase in the amount of lipid droplets in similar cells of R. pipiens (Burgos, 1951, 1952, 1955).

Amphibian gonadotrophins are probably similar to those of the mammals. Both exogenous Xenopus A.L.P. and ovine A.L.P. will stimulate the development of gloves in hypophysectomised male X. laevis and thumb pad development has been reported in juvenile B. fowleri after injection of H.C.G. (Berk, 1939; Blair, 1946). Similarly, Amphibian pituitaries have gonadotrophic activity when injected into mammals (Novelli, 1932; Zwarenstein, 1939, 1942).

2. Female Amphibia.

The effect of hypophysectomy on the ovaries.

Hypophysectomy of immature female Urodeles prevents the development of the ovaries and secondary sex characters. In adult Urodeles it causes the regression of the ovaries and eventual degeneration of the secondary sex characters (Woronzowa and Blacher, 1930; Burns and Buyse, 1932).

A similar situation is found in the Anura. In X. laevis, adenohypophysectomy results in atrophy of the ovaries to an embryonic appearance with no visible ova (Hogben, 1930; Hogben, Charles and Slome, 1931; Shapiro and Shapiro, 1934; Bellerby and Hogben, 1938). The latter authors showed that the lowered metabolic rate and reduced food intake which occurs after adenohypophysectomy is not the cause of ovarian regression. Gallien (1939, 1940) found that hypophysectomy during the summer prevented normal ovarian vitellogenesis in R. temporaria. However, in frogs hypophysectomised in December and killed in February, the ovaries and oviducts were not noticeably different from those of normal controls. Ten months after hypophysectomy, however, a new crop of eggs failed to develop. Gallien found that the presence of the pituitary is not necessary for the initial development of the oöcytes.

The effect of exogenous gonadotrophins.

Homo-implantation of the pars distalis or injections of pituitary extracts stimulates ovulation in normal B. arenarum and restores to normal the atrophied ovaries of hypophysectomised Bufo spp. (Houssay, Giusti and Lascano-Gonzalez, 1929; Houssay and Giusti, 1930; Houssay, 1949). Mature R. pipiens and Rana sphenocephala ovulate in response to injection of frog pars distalis (Wolf, 1929; Wills, Riley and Stubbs, 1933).

Hogben (1930) found that oviposition and ovulation occurred in X. laevis after injection of mammalian pituitary gonadotrophin. This observation stimulated research on the effect of gonadotrophins upon ovulation and oviposition in other Anura. Reviewing work up to that date, Rugh (1935) reported that ovulation, but not oviposition, had been stimulated in over 20 species of Amphibia by injections of gonadotrophins of Amphibian or Mammalian origin. When ovulation occurs in most Anura it is not followed by oviposition. Ovulated ova are stored in the non-ciliated caudal end of the oviduct which forms the ovisac. Ova remain there until coupling occurs when oviposition follows. X. laevis is exceptional in that the oviducts are ciliated throughout their length and oviposition automatically follows ovulation (Waring, Landgrebe and Neil, 1941). One other species, B. bufo gargarizans, has been reported to oviposit and ovulate

after being injected with H.C.G. (Ying-tien, 1963).

Other Anuran species ovulate, but do not oviposit, in response to exogenous gonadotrophin with luteinising activity. Literature on the comparative effects of F.S.H. and L.H. on the ovaries of hypophysectomised Amphibia is scarce. Waring, Landgrebe and Neil (1941) found that injection of pregnancy urine and mammalian pituitary extracts stimulates enlargement of the reduced ovaries of adenohypophysectomised X. laevis. Gitlin (1941) found that ovine pituitary gonadotrophin, injected into intact X. laevis during November, causes an increase in the weight of the ovaries, which are then normally small. Recently hypophysectomised R. pipiens have been reported to ovulate when injected with frog pituitary but to be insensitive to a purified preparation of ovine pituitary F.S.H. (Wright and Hisaw, 1946). There is some evidence that F.S.H. sensitizes the ovary to L.H. Although X. laevis do not ovulate in response to exogenous F.S.H., animals which have failed to ovulate after repeated injections of H.C.G. become sensitive to H.C.G. after an injection of P.M.S. (Hobson, personal communication).

<u>The control of the female secondary sex characters.</u>

Tuchmann-Duplessis (1945) found that the nuptial array of female Triturus spp. disappears more rapidly after hypophysectomy than after ovariectomy. He postulated that the pituitary has a direct action on some

Urodele secondary sex characters. However, his evidence does not preclude a pituitary effect mediated by the adrenal cortical tissue. Smith (1955), reviewing reproduction in female Amphibia, concluded that amphibian secondary sex characters are controlled by the gonads.

(i) The effect of ovariectomy.

If Triturus cristatus are ovariectomised during the breeding season, when the cloaca is swollen, the swelling regresses (Bresca, 1910). The Anuran female secondary sex characters comprise the oviducts, areas of ciliated peritoneum and, in X. laevis, large cloacal labia. The passive behaviour of some female Anura during amplexus can also be regarded as a secondary sex character as the mating reflex is reported to disappear after ovariectomy of R. temporaria (Shapiro, 1937). This author found that hypophysectomy causes a similar loss of the female mating reflex which can, however, be restored by injecting frog A.L.P. The failure of exogenous A.L.P. to restore the mating reflex in ovariectomised frogs confirms the ovarian control of this reflex (Shapiro, 1937).

The most noticeable effect of ovariectomy is upon the oviducts. Ovariectomy of R. pipiens during September is followed by marked degeneration of the oviducts and loss of glandular activity within 3 months, and ovariectomy of mature R. pipiens causes complete regression of the oviducts to the larval condition within 8 months (Wolf, 1928;

Christensen, 1931). Ovariectomy of juvenile and adult B. arenarum has shown that the oviducts and their glands develop in the absence of the ovaries but in adult toads ovariectomy is followed by a slow regression of the oviducts (Houssay, 1952). Ovariectomy or adenohypophysectomy causes a decrease in the weight of X. laevis oviducts (Gitlin, 1939). It seems to be generally agreed that the seasonal development of the Amphibian oviduct is controlled by ovarian hormones, but some workers believe that secretion by the oviducal glands may be under other control.

(ii) the effect of injected hormones.

Injection of female X. laevis with bovine pituitary gonadotrophin or H.C.G. causes them to be clasped by males (Shapiro, 1936a; Russell, 1954). Shapiro believed that exogenous gonadotrophin causes a passive behaviour in female Xenopus, but Russell found that uninjected females are no less passive than injected ones but that they emmit a "ticking" sound when males attempt to clasp them whereas uninjected females remain silent. It appears that ticking inhibits the maintenance of the clasp by the male.

Gitlin (1939) reported a seasonal variation in the weight of X. laevis ovaries and oviducts. He found that injection of mammalian pituitary extracts will cause an out-of-season increase in oviduct weights in normal and hypophysectomised Xenopus. He postulated a pituitary-gonadal control of oviduct weight.

In R. pipiens, the injection of oestradiol causes hypertrophy of the Müllerian ducts of both male and female newly-metamorphosed frogs. This hormone increases the size of the oviducts of ovariectomised adults (Wolf, 1939; Schreiber and Rugh, 1945). Blair (1946) found only slight hypertrophy of the oviducts of juvenile B. fowleri after injecting α-oestradiol benzoate and testosterone. Ponse (1941) found that injection of oestrone had no effect on the atrophied oviducts of ovariectomised B. bufo (B. vulgaris), whereas homo-implantation of ovaries caused their regeneration. She concluded that the Bufo ovary secretes an oestrogen different from those of the Mammalia.

There is evidence which suggests that secretion of the Anuran oviduct is under pituitary control. For example homo-implantation of partes distales or injection of mammalian pituitary extracts cause oviducal secretion in both normal and ovariectomised B. arenarum (de Allende, 1939). In this species, injection of oestrogens causes little or no oviducal secretion (de Allende, 1939; Houssay 1952). However, the non-steroid di-ethylstilboestrol has been found to cause secretion of the oviducts when injected into T. viridescens (Adams, 1950).

An ovarian control of oviducal secretion is suggested by the report of Blair (1946) that injections of toad A.L.P. will only stimulate the development of large convoluted oviducts in juvenile B. fowleri which have large mature ovaries. Galli-Mainini (1950, 1951) found

that exogenous gonadotrophin stimulates oviducal secretion in normal but not in ovariectomised B. arenarum.

de Allende and Orias (1955) have shown that the ova themselves may be important in stimulating oviducal secretion. They occluded one oviduct with a ligature and injected the toads with H.C.G. They found that secretion only occurred in the unobstructed oviduct through which the eggs passed. de Allende and Orias (1955) concluded that this secretion may be partly stimulated by the passage of the ova. They did not, however, try the effect upon oviducal secretion of eggs introduced into the body cavity of uninjected toads.

Oviducal secretion has been stimulated in castrated and hypophysectomised B. arenarum by injections of prolactin and progesterone (Houssay, 1952). Lodge and Smith (1960) obtained an ovarian extract from B. bufo which causes secretion when injected into the lumen of the oviducts in B. bufo and R. temporaria. Basu, Bern and Chang (1965) injected purified prolactin into R. pipiens and Bufo americanus during the summer when endogenous stimulation of the oviducts is very low. They found no oviducal secretion after 14 daily injections. The evidence for prolactin control of secretion in the Anuran oviduct is therefore contradictory.

The hormonal control of oviducal secretion in Urodeles is based upon histochemical evidence in the newt

Triturus pyrrogaster. The oviducts of this species alter little in general structure during the breeding season, but the distribution and activity of alkaline phosphatase in the oviducal epethelium is different during the breeding season from that before the breeding season. Before the breeding season, the cytoplasm of the oviducal epethelial cells exhibit no alkaline phosphatase reaction and the nuclei only a weak to medium reaction. As the breeding season approaches, both cytoplasm and nuclei show strong alkaline phosphatase reactions (Kambara, 1956a,b, 1963). Kambara (1964) suggested that alkaline phosphatase is involved in the secretion of oviducal jelly. He found that an increase in alkaline phosphatase activity could be stimulated in the oviducts of normal, but not of ovariectomised newts, by injecting newt pituitaries or P.M.S. (Kambara, 1963a). This result supports the theory that pituitary gonadotrophins indirectly stimulate oviducal secretion in the Amphibia by stimulating secretion of an ovarian hormone. There is some evidence that this hormone is progesterone. Injections of progesterone stimulate an increase in alkaline phosphatase activity in normal, ovariectomised and ovariectomised plus hypophysectomised newts (Kambara, 1962, 1963a, 1964).

Exogenous oestriol, oestrone, testosterone and deoxycorticosterone acetate appear to stimulate an increase in the weight of the oviducts of ovariectomised newts but to

have little or no stimulatory effect on alkaline phosphatase activity. Oestriol and Oestrone inhibit the response to progesterone (Kambara, 1964).

These experiments indicate that during, or just before, the breeding season the newt ovary secretes a progestogen and an oestrogen, which respectively stimulate secretion and hypertrophy of the oviducts.

Other Anuran female secondary sex characters have also been found to be under hormonal control. The effect of ovariectomy on the peritoneal cilia does not appear to have been investigated, but injection of oestrone has been found to cause some ciliation in male R. pipiens (Donahue, 1934). As intact frogs were used, there is no evidence that the effect of oestrogen was directly on the peritoneum.

The cloacal labia of female X. laevis become swollen, turgid and highly vascularized during the spring (Bles, 1905). This phenomenon is known as "cloacal hyperaemia". It has been stimulated in the intact female by injection of Xenopus pars distalis, mammalian pituitary gonadotrophin, H.C.G., progesterone and testosterone (Zwarenstein, 1936; Shapiro, 1936; Shapiro and Zwarenstein, 1937; Berk and Shapiro, 1939). Shapiro and Zwarenstein (1937) found that cloacal hyperaemia could not be induced in ovariectomised X. laevis with injections of mammalian pituitary extract, H.C.G., progesterone, testosterone, oestrone or oestradiol. They concluded that cloacal hyperaemia is

caused by an ovarian hormone different from mammalian sex hormones.

(iii) The effects of hypophysectomy.

The effect of the pituitary on the development and maintenance of the Amphibian secondary sex characters is shown by the effects of hypophysectomy. Hypophysectomy of 3 species of immature Urodeles has been found to prevent the development of the ovaries and secondary sex characters. In adult animals atrophy of the ovaries and regression of the secondary sex characters follows hypophysectomy within 3 years (Woronozowa and Blacher, 1930). Similar results have been reported in Ambystoma tigrinum (Burns and Buyse, 1932). In adult R. pipiens, degeneration of the oviducts follows within eight months of hypophysectomy (Christensen, 1931). In R. temporaria, regression of the oviducts after hypophysectomy is slow, with the rate of atrophy depending on the time of year when hypophysectomy is performed (Gallien, 1939, 1940). In this species, removal of the pars distalis before the breeding season prevents the appearance of the mating reflex (Shapiro, 1937).

Bellerby and Hogben (1938) found only a slight regression of the oviducts of X. laevis, but definite histological changes in the oviducal glands, after hypophysectomy. Neither hypophysectomised nor ovariectomised female X. laevis are clasped by sexually active males

(Shapiro, 1937; Weisman and Coates, 1944). Weisman and Coates assumed that failure by the males to clasp is due to a change in the appearance of hypophysectomised females when their ovaries atrophy. However, it seems probable that hypophysectomised females emmit the ticking sound heard from females which fail to couple (Russell, 1954). The possibility that a female with mature ovaries secretes a pherome which attracts the male does not appear to have been investigated.

The activity of steroids in inducing ovulation.

The first evidence that gonadotrophins may only be indirectly involved in ovulation was provided by Zwarenstein (1936) who found that injecting progesterone causes ovulation in both intact and hypophysectomised X. laevis.

Shapiro (1939) injected female X. laevis with over 30 steroids. He reported ovulation in response to progesterone, testosterone, androsterone, transdehydroandrosterone, androstenidione, cisandrostane-2,17-diol, allopregnanedione, allopregnanetriol and "eucortone", in the intact animal and in response to progesterone and testosterone in hypophysectomised toads. Shapiro found that oestriol, oestradiol glucuronide and pregnanediol do not cause ovulation when injected into normal females. He concluded that there is no specific steroid configuration which can be associated with gonadotrophic properties in

X. laevis. The author stated that this work was purely qualitative but it must be criticized on the grounds that structure-action relationships were discussed when evidence of inactivity of some substances was very slender. No attempt was made to ensure that the toads used were capable of ovulating, and many substances were injected into only one toad.

Sapeika (1943) found that deoxycorticosterone acetate (D.O.C.A.) and progesterone have similar activity in causing ovulation in mature X. laevis. He found that 25 out of 38 toads with good ovaries ovulated in response to 330 μg D.O.C.A. and 16 out of 25 toads to the same dose of progesterone. Sapeika killed his animals after the experiments, and discarded 12 toads with atrophic ovaries. The existence of such animals strengthens the criticisms of the work of Shapiro (1939).

Progesterone and testosterone have been found to cause ovulation when injected into intact R. pipiens, but α-oestradiol dipropionate was inactive (Langan, 1941).

The finding that injection of eucortone causes ovulation in X. laevis is confirmed by the induction of ovulation with pure adrenocorticosteroids. Corticosterone, 11-deoxycorticosterone, hydrocortisone (cortisol), cortisone, 17-hydroxy-11-deoxycorticosterone and aldosterone have all been reported active in this respect (Burgers and Zwarenstein, 1955; van der Schyff and Zwarenstein, 1962).

Simultaneous injection of cortisone has been reported to sensitize R. pipiens and Rana cyanophlyctis to exogenous frog pituitaries (Chang and Witschi, 1957; Ramaswami and Lakshman, 1958) but to be ineffective in causing ovulation in R. pipiens when injected alone (Chang and Witschi, 1957).

Injection of progesterone and methyl testosterone cause ovulation in R. cyanophlyctis (Ramaswami and Lakshman, 1958) and in R. pipiens (Wright and Flathers, 1961).

Ying-tien (1963) reported ovulation in B. bufo gargarizans in response to injected progesterone and pregnanediol, but not to 4 mg. cortisone acetate, 2 mg. testosterone or oestradiol benzoate (dose not stated in the English summary). He also found that simultaneous injection of unspecified steroids caused ovulation when given with a sub-threshold dose of H.C.G. or toad pituitary. An interesting finding was that exogenous gonadotrophin stimulates oviposition as well as ovulation in this species.

Ovulation in-vitro.

In-vitro methods have been used to discover which substances have a direct action upon the excised ovary. In-vitro ovulation of excised R. pipiens ovaries, or pieces of ovary, in Ringer's solution containing frog pituitaries has been reported by Heilbrunn, Daugherty and Wilbur (1939) and by Ryan and Grant (1940). In-vitro

ovulation has been reported in response to Anuran and Mammalian gonadotrophins by Grant (1940), McPhail and Wilbur (1943), Wright (1945, 1949, 1950), Wright and Hisaw (1946) and Burgers and Li (1960) but these workers used Holtfreter's solution, in some cases diluted to 10%, or water. These fluids are all hypotonic to adult Anuran body fluids, and as hypotonic Xenopus saline causes in-vitro ovulation of Xenopus ovaries (see page 130 of this thesis), these results must be treated with reserve.

Chang and Witschi (1957) found that cortisone caused the in-vitro ovulation of ovaries removed from R. pipiens which had been injected with a sub-threshold dose of gonadotrophin. These ovaries would not ovulate in plain Ringer's solution. Chang and Witschi postulated that cortisone exerts a "last step" effect on ovulation.

Wright (1961a,b) reported that excised R. pipiens ovaries ovulate in Ringer's solution containing various steroids. However, he "sensitized" the ovaries used in-vitro by injecting the frogs with pituitaries 16 hours before removal of the ovaries, so the substances reported as causing ovulation essentially only augmented the effect of injected gonadotrophin. An interesting observation made by Wright was that oestrone, either injected with the sensitizing pituitary or added with pituitary in-vitro, antagonised the effect of gonadotrophin.

Edgren and Carter (1961, 1963) experimented with small fragments of R. pipiens ovaries in Petri dishes of

10 ml. Ringer's solution. They reported that progesterone causes in-vitro ovulation but that the response falls off with doses over 300 µg./ml. The in-vitro ovulation response to progesterone was found to be inhibited by H.C.G., cortisone, deoxycorticosterone, and 19-nortestosterone but not by oestrone, oestradiol, hydrocortisone or testosterone. Edgren and Carter did not find the potentiation by oestrone and oestradiol reported by Wright (1961b) and ascribed this to a difference in response between the ovaries of their frogs which were excised in July and those of Wright which were removed in November. Edgren and Carter (1961) found that H.C.G. administered alone in doses between 0·03 and 3·0 I.U. stimulate in-vitro ovulation.

De Corral (1959) tested the activity of a number of steroids upon the excised ovaries of B. arenarum. Some augmented the activity of pituitary material both when injected in-vivo and when added with the pituitary in-vitro. The only steroids tested by themselves in-vitro were progesterone and nor-ethinyl testosterone; they did not cause ovulation.

Ovulation of excised ovaries of B. bufo gargarizans has been studied by Chang and Tsaung (1963) and by Ying-tien (1963). Only the summaries of their papers have been available in English so they cannot be extensively discussed.

Ying-tien (1963) found that H.C.G., progesterone,

allopregnanediol and testosterone induce in-vitro ovulation and also augment the response to toad pituitary. Oestradiol benzoate did not induce ovulation.

Chang and Tsaung (1963) suspended fragments of ovaries from toads hypophysectomised 3-6 days previously in 20 ml. Ringer's solution and found that ovulation is induced by the addition of progesterone, testosterone, cortisone and deoxycorticosterone. Androsterone, hydrocortisone, dehydroepiandrosterone and adrenosterone only augmented the effect of a sub-ovulatory dose of toad pituitary. It is interesting that Chang and Tsaung also found that growth hormone (G.H.) and L.H. were also inactive alone but would augment the action of a non-ovulating dose of toad pituitary. These substances were found to cause in-vitro ovulation by Burgers and Li (1960) who used ovaries from R. pipiens which had been "sensitized" by a previous injection of pituitary. It seems probable that their results might have been different if they had not "sensitized" the ovaries.

In X. laevis, in-vitro ovulation has been reported in response to progesterone, testosterone, androstenidione, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, hydrocortisone, and cortisone (Shapiro and Zwarenstein, 1937; Zwarenstein, 1937; Burgers and Zwarenstein, 1955).

Isolation of steroids from Amphibia.

Progesterone has been reported in the blood of several species of frogs (Lazo-Wasen, Neher, Shoger and Zarrow, 1954). Cortisone has been found in blood collected from the posterior vena cava of X. laevis (Phillips and Chester Jones, 1957).

Chieffi and Lupo (1963) extracted the ovaries of B. bufo and estimated the concentrations of oestriol, oestradiol-17β, oestrone and progesterone to be 60 μg., 40 μg., 41 μg., and 16 μg. per kg. respectively. It is not stated whether these concentrations relate to wet or dry tissue.

There is evidence of progesterone and oestradiol-17β in the testes of B. bufo and T. cristatus carnifex (Chieffi and Lupo, 1961; Della Corte and Cosenza, 1965).

In larval X. laevis, before and during metamorphosis, the gonads have been found to contain traces of oestrone and oestradiol. Eleven months after metamorphosis the amounts were 7 and 12 times higher at 0·040 μg. and 0·060 μg. per animal respectively. After injection of H.C.G. these concentrations were doubled in 14 days for oestrone and in 14 hours for oestradiol (Gallien and le Foulgoc, 1960).

Larval R. pipiens and Rana sylvatica have been found to excrete hydrocortisone, and probably traces of aldosterone, androsterone and oestradiol-17β (Dale, 1962).

Dale postulated that androsterone and oestradiol are probably secreted by the gonads, but he does not exclude a possible adrenal origin.

Pesonen and Rapola (1962) histochemically examined Amphibian tissues for steroid-3β-ol-dehydrogenase, the presence of which indicates steroid synthesis. This enzyme was found in the adrenals of male and female B. bufo and X. laevis after administration of H.C.G., P.M.S. or adrenocorticotrophic hormone (A.C.T.H.), and in the testes of X. laevis after treatment with A.C.T.H. No steroid-3β-ol-dehydrogenase could be found in Bufo ovaries and testes or in Xenopus ovaries after treatment with 50 I.U./day P.M.S. and H.C.G.

Other workers have used in-vitro techniques to investigate the ability of Amphibian tissues to metabolise steroids added to the culture medium. It has been found that ovarian and testicular tissue of R. temporaria rapidly metabolise testosterone, the testes at twice the rate of the ovaries (Ozon, Breuer and Lisboa, 1964). Pleurodeles waltlii testes have been found to synthesise testosterone, and the ovaries to synthesise oestrone and oestradiol-17β from carbon-14 labelled progesterone (Ozon, 1967). Ozon found that, when the ovaries contain mature ova, the metabolism of progesterone is similar to that in the testes, going through the sequence 17-hydroxyprogesterone $\longrightarrow$ androstenidione $\longrightarrow$ testosterone. Reductive metabolism of testosterone has been found to occur in the

liver of P. waltlii and R. temporaria and to be similar to that in the Mammals (Lisboa and Breuer, 1966).

Ozon and Breuer (1966) have also demonstrated the ability of P. waltlii to conjugate steroids to form glucuronides. They added oestrone to the water in which the newts were kept and detected oestradiol and oestrone glucuronides in the water within 40 hours. The enzyme glucuronyl transferase, which is involved in gluronidation, was detected in the microsomal fraction of homogenates of P. waltlii livers.

The ovaries of R. pipiens synthesise principally testosterone from progesterone added to the culture medium, and those of Necturus maculosa synthesise androstenidione (Callard and Leatham, 1966).

Evidence for the occurrence of steroids in Amphibia is accumulating; but the evidence of some workers is opposed to that of others. Personen and Rapola (1962) suggest that there is no appreciable production of steroids in X. laevis ovaries, whereas Gallien and le Foulgoc (1960) found both oestrone and oestradiol in them. Hobson (personal communication) has demonstrated oestrogenic activity in powdered dessicated Xenopus ovaries using vaginal cornification in the ovariectomised mouse as the index of response. Personen and Rapola (1962) also stated that oestrogens never stimulate ovulation in-vitro; this is at variance with results reported in this thesis.

The influence of external factors on reproductive activity.

Many Amphibia breed once a year, but spawning times vary. This variation makes it likely that reproductive processes are influenced by the environment.

Bles (1905) induced oviposition in X. laevis by raising the water temperature and simulating rainfall; rainfall and temperature have also been found to affect the date of spawning in R. temporaria (Savage, 1935). Marshall (1936, 1942) suggested that external factors exert their influence by nervous stimulation of the release of gonadotrophic hormones by the anterior lobe of the pituitary.

In X. laevis, the onset of coupling has been correlated with a sharp rise in temperature and a high rainfall, and the regeneration of the ovaries, which occurs earlier, with a period of increasing rainfall and decreasing temperature (Berk, 1938). Bellerby (1938) found that the intensity and wavelength of light appeared to have no effect on the sexual cycle and concluded that the sexual cycle is related to changes in water volume, temperature and nutrition. His conclusions were partly confirmed by Alexander and Bellerby (1938) who found that ovarian activity in X. laevis ceases when the ponds, in which they live, dry up at the end of the breeding season.

Some effects of environment on reproduction have been reported in R. esculenta and T. cristatus (Galgano, 1931, 1932, 1934, 1935, 1936, 1941, 1947). Significant

findings were that high temperature in winter causes atrophy of the thumb pads, and that in R. esculenta - which has been described by van Oordt (1960) as a potential continuous breeder - spermatogenesis can always be induced by raising the temperature and inhibited by lowering it.

Seasonal cytological changes in the pars distalis of Rana spp. have been reported (Zahl, 1937) and a seasonal variation in weight of the pituitary has been found in R. pipiens (Grant, 1940).

In R. temporaria, temperature has been found to affect the gonadotrophic activity of the pars distalis (van Oordt, 1956). Keeping frogs at summer temperatures during October provokes an increase in F.S.H. activity and a decrease in I.C.S.H. activity as measured by histological changes in the β- and γ-cells of the pars distalis, accompanying changes in the Sertoli and interstitial cells of the testes and changes in the thumb pads (van Oordt and Lofts, 1963).

An "inherent" cyclical variation in the sensitivity of the primary spermatogonia to gonadotrophin has also been reported (van Oordt, 1956, 1960); whether this variation is influenced by environmental changes does not seem to be known.

There seems little doubt that the gonadotrophic activity of the pars distalis is influenced by environmental factors, and there is considerable evidence that

the hypothalamus is involved in the control of the mammalian pituitary (Harris, 1955). Evidence of a similar control in the Amphibia is accumulating. Chemical and electrical stimulation of the hypothalamus of male B. arenarum has been found to cause spermiation (Astrada, 1962). This effect cannot be ascribed to a liberation of gonadotrophin as occurs during hypophysectomy (Wright and Hisaw, 1946; Wright, 1949), as mechanical stimulation of the hypothalamus was found to have no effect.

The morphology, histology and cytology of Amphibian pituitaries have been described by Herring (1913), Rimer (1931), Levenstein and Charipper (1939), Atwell (1941), Charipper and Martorano (1948), Zuber-Vogelli (1952), Cordier (1953), van Oordt (1961, 1963), Doerr-Schott (1965) and Kerr (1965). Attempts to correlate function with particular cell types has been bedevilled in the past by a lack of uniformity in the nomenclature of adenohypophyseal cells. An attempt has recently been made to correlate names given by different workers and to name cells according to their function (van Oordt, 1965). As, however, little is known with certainty about which cells secrete which hormones in many vertebrates, the proposed names appear applicable only to the Mammals at the present.

In the pars distalis of R. temporaria, B. bufo and X. laevis van Oordt (1961, 1963) ascribed the secretion of F.S.H. to cells he named β-cells and ascribed the

secretion of L.H. to cells he termed γ-cells. The hyperactivity of the β-cells after castration and their hypoactivity after administration of testosterone in male R. temporaria is taken as evidence that these cells secrete F.S.H. Similarly, the increase in the number of γ-cells after gonadectomy, and the correlation between the regression of these cells and the secondary sex characters of frogs kept at high temperatures, indicates that the γ-cells secrete L.H. Two similar types of cells have been described in the pars distalis of X. laevis by Kerr (1965). He found that gonadectomy caused changes in a cell which appears to be equivalent to the β-cells of van Oordt and he ascribed secretion of F.S.H. to these cells.

The structural relationship between the hypothalamus and the hypophysis has been investigated in R. temporaria by Dierickx (1964a,b, 1965, 1966a,b). He found that proper gonadotrophic function of the pars distalis depends upon a normal relationship between it and the hypothalamus. He demonstrated that removal of the median eminence in June, when the gonads are small and the secondary sex characters minimal had the same effect of preventing normal development of testes, thumb pads, ovaries and oviducts as did removal of the pars distalis. He also showed that transfer of the pars distalis from its normal site to other positions in the body caused the same

effects in 58 out of 61 frogs, (Dierickx, 1964a).

He was able to show that cells of the median eminence of the hypothalamus are probably involved in the liberation of gonadotrophin releasing factors into the hypothalamico-hypophyseal portal system, by removing the nervous tissue from the median eminence of female frogs while leaving the portal system intact (Dierickx, 1964b). In those frogs, in which the nerve fibres did not regenerate, the seasonal development of the oviducts and ovaries was prevented. Dierickx (1965) later demonstrated that particular nerve fibres, the cell bodies of which form nuclei in the hypothalamus, are responsible, via the pars distalis, for seasonal development of the ovaries, eggs and oviducts in the female and seasonal development of the thumb pads in the male. Similar experiments were reported which indicates that the pars ventralis of the tuber cinereum of the hypothalamus is an important gonadotrophic centre responsible for the seasonal development of the testes in the male R. temporaria and ovulation in the female (Dierickx 1966a,b).

A dependence of the pars distalis on the hypothalamus has been demonstrated in B. bufo by Jørgensen (1963) who found that the gonadotrophic function of the pars distalis was too low to maintain mature eggs in the female when its connection with the hypothalamus was severed. This dependence was elegantly shown by van Dongen, Jørgensen,

Larsen, Rosenkilde, Lofts and van Oordt (1965) who compared the effects of removal of the pars distalis with autotransplantations of the pars distalis into an ectopic position and on to the median eminence. They reported that homoiotopic transplantation maintained almost normal spermatogenesis, interstitial cell activity and well developed thumb pads. Ectopic transplantation resulted in only slightly reduced spermatogenesis but a reduced activity of the interstitial cells and regression of the thumb pads. These effects occurred more rapidly after adenohypophysectomy. Results were less clearly defined in females. In female frogs operated upon in the autumn, all those with ectopic grafts, and some of those with homoiotopic grafts, had atrophic ovaries. van Dongen et al. (1965) concluded that ectopic transplantation of the pars distalis prevented it from receiving normal amounts of releasing factors via the portal system, but that the continued gametogenesis implied a continuing secretion of F.S.H. by the ectopic pars distalis. They further concluded that the cells of the pars distalis which secrete F.S.H. are not so dependent upon a releasing factor.

Evidence is accumulating that environmental conditions influence the activity of the pars distalis via the hypothalamus. It is also probable that there is a negative feed-back mechanism by means of which an increase

in steroid secretion by the gonads depresses pituitary gonadotrophin secretion. However, there is little evidence to show if this effect is directly upon the pituitary or via the hypothalamus.

Few attempts have been made to assay the gonadotrophic activity of the Amphibian pars distalis under different conditions. Novelli (1932) assayed the gonadotrophic activity of the B. arenarum pituitary at various times after castration and was not able to distinguish any change up to 90 days after castration. He used the number of ovulations in female toads injected with pituitary extracts as the biological response.

Grant (1940) assayed the gonadotrophic activity of R. pipiens pituitaries at different times of the year; the index of response being induction of ovulation in-vitro. He found that pituitaries of frogs which had just ovulated were half as potent as those from frogs killed during hibernation; potency increased in late September and at ovulation.

van Oordt (1951, 1952) found a difference between the gonadotrophic activities of pituitaries taken from frogs in summer and in winter. He assayed the gonadotrophic activity of pituitaries in terms of their potency in stimulating spermatogenesis when injected into male frogs.

The effect of exogenous steroids upon the gonadotrophic activity of the Amphibian pars distalis does not

appear to have been investigated.

Induction of anaesthesia in X. laevis.

At the beginning of this investigation it became evident that, whatever anaesthetic was used during operative procedures, it was essential to have some knowledge of any effects it might have on X. laevis which might interfere with the results of the operations themselves.

Di-ethylether (ether) and ethyl carbamate (urethane) have been commonly used to anaesthetize laboratory animals. Ether in high concentrations acts rapidly but causes stress in the animal, and its inflammability precludes its use with cautery. Aquatic vertebrates may be anaesthetized by injection or immersion in an aqueous solution of urethane. Although urethane has a more moderate action on the organism, it has been found to have carcinogenic properties (Ball and Cowen, 1959; Revici, 1961).

Experience with tricaine methane sulphonate (M.S. 222 Sandoz) has suggested that it might be the ideal anaesthetic for poikilothermic vertebrates; both larval and adult Urodeles and Anurans have been successfully anaesthetized with tricaine (Sandoz, 1959). However, detailed information about the response of individuals injected with, or immersed in, solutions of tricaine is available for only a few species of vertebrates.

The suitability of tricaine as an anaesthetic for X. laevis has been compared with that of ether and urethane. The effects of temperature, repeated use, and body weight upon the times taken for anaesthesia and recovery have been investigated, together with certain other aspects such as stress, measured in terms of blood sugar level, and spermiation.

EXPERIMENTAL PROCEDURE.

Husbandry of Xenopus laevis.

Contrary to early reports (Zwarenstein and Shapiro, 1933; Shapiro and Shapiro, 1934), X. laevis may be kept in the laboratory without ill effect, but it is important to carefully control the conditions under which they are kept.

An adequate intake of food is necessary, and, as overcrowding causes a reduced food intake and regression of the ovaries, it is important to avoid overcrowding (Alexander and Bellerby, 1935, 1938). Pollution due to overcrowding can be a major factor in reducing food intake, and it has been suggested that an interaction between the toads results in fatigue due to competitive effort before much food has been eaten (Landgrebe, 1939).

Except where otherwise stated, all the toads used in the experimental work reported upon in this thesis were kept under the following conditions.

Stock toads were kept in groups of 100, of one sex, in large fibre-glass tanks containing 160 litres of water. The rate of water flow was adjusted to cause a daily complete change of water. During short-term experiments toads were isolated in square, screw-topped glass jars containing 0·75 L. water. During longer experiments toads were kept in small tanks containing 7·5 L. water and up to 10 toads; this was necessary in order to get them

to feed. These toads were identified by claw-clipping, the claws being re-cut when necessary in long-term experiments. By cutting up to 2 claws, 22 toads could be identified.

The temperature of the water in which toads were kept was maintained at 22°C ± 1°C, the temperature found suitable for X. laevis by Landgrebe (1948).

Toads were fed at least once a week on fresh ox-liver chopped into cubes of about ¼ inch square; this is an ideal food (Landgrebe, 1939). As far as was possible, toads were not fed within 2 days before or after an injection or operation. This was because disturbance beforehand tends to prevent their feeding and injection soon after feeding can cause vomiting. Particular care was taken to re-establish feeding after adenohypophysectomy, as, once this was achieved, adenohypophysectomised toads survived for months. All uneaten food was removed from the tanks on the morning after feeding; in the small tanks the water was changed on each of the 2 days following feeding.

Handling and injection.

X. laevis are very slippery to hold and were caught and held by a special technique. When the hand is put gently into a tank, toads tend to come towards the hand apparently expecting food. The forefinger is then put gently between a toad's hind legs which are then

firmly grasped (Plate I).

Injections were made into the dorsal lymph sac (d.l.s.) by the method of Landgrebe (1948). The toad is caught with one hand and transferred to the other hand and held firmly by its hind legs in a dry cloth (Plate II). The point of the needle, bevel uppermost, is passed through the thigh muscle, which acts as a valve, tilted upwards, and directed towards the mid-line of the dorsal surface just under the skin (Plate II). Care is taken to ensure that the needle is free in the lymph sac; the fluid can then be seen to flow freely towards the head when the toad is tilted as the injection is made.

Unless otherwise stated, substances were injected dissolved or suspended in 1 ml. distilled water. The finest possible suspension was made of substances not soluble in water by grinding them in an all-glass tissue grinder. Methyl cellulose was tried as a suspending medium but was found to be toxic to Xenopus when injected in a concentration sufficient to suspend water-insoluble steroids.

Operative Techniques.

Anaesthesia.

All operations were performed with the toads anaesthetised by injection with a solution of tricaine methane sulphonate (MS 222, Sandoz).

An investigation of the suitability of the drug as an

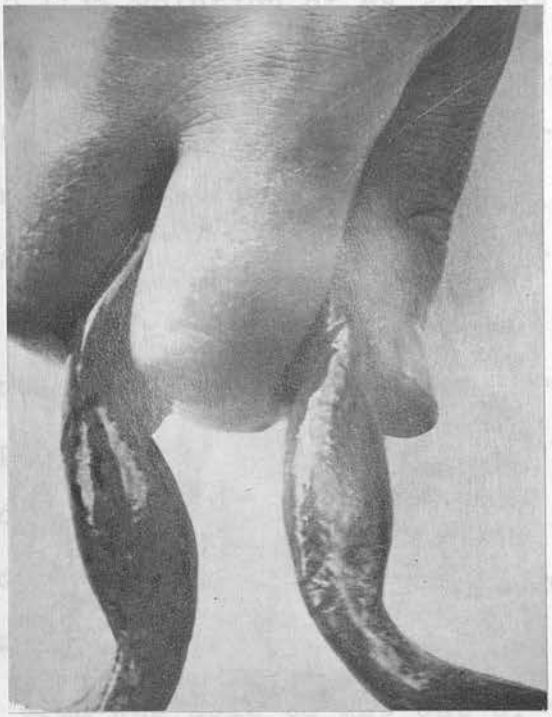

PLATE I.

The method of holding Xenopus.

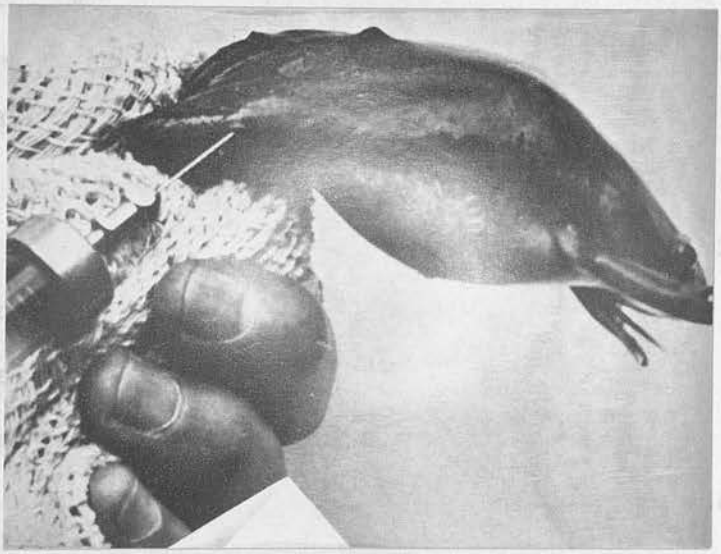

PLATE II.

Injection of Xenopus.

anaesthetic during operations performed during this investigation is reported in this thesis.

A fresh 1·3% solution of MS 222 was prepared before each series of operations and kept in a refrigerator during the series. Using a 1 ml. tuberculin syringe graduated in 0·01 ml. divisions, 0·01 ml. of a 1·3% solution was injected for each gramme of body weight.

Whilst recovering from anaesthesia, each toad was put into running water and observed. This was conveniently done by putting the toad into a jar which was laid on its side and slightly tilted; the toad lay with its nostrils above water. Water was arranged to run into, and overflow from, the mouth of the jar. I found that as soon as the toad was able to get out of the jar, it was able to swim and could then be returned to its tank.

Gonadectomy.

Male X. laevis were castrated by making a median incision through the skin of the ventral body wall. The muscle wall was then divided by a para-median incision. This avoids the anterior abdominal vein, and by offsetting the inner and outer incisions, prevents the later development of adhesions between the two incisions.

Both testes were removed, and the fat bodies left in the body. In Xenopus, each testis is attached to the ventral surface of the lateral edge of the kidney by a mesentery which contains the arteries, veins and sperm

ducts. It was found that practically no bleeding occurs when this mesentery is simply cut with scissors taking care to leave intact the blood supply to the fat bodies.

Ovaries were removed in the same way except that a cautery knife was used to separate the mesentery joining each ovary to the kidney. This prevented bleeding from the large blood vessels.

All inner and outer incisions were sutured with 4/0 chromic catgut; the wounds healed very well, even in toads which had also been adenohypophysectomised (Plate III).

Adenohypophysectomy.

In the Anura, the pituitary is dorsal to a depression in the parasphenoid bone and the route of approach was through the mouth.

The toad was anaesthetised and laid on its back. The mouth was held open by retractors adapted from a pair of forceps. These were shaped so that one arm fitted into one of the internal nares and the other fitted against the lower side of the buccal cavity. The tissues were protected from damage with a specially shaped piece of card which was inserted into the mouth. The screw of the retractors was adjusted to hold open the mouth without unduly stretching the jaw ligaments.

A median incision was made in the roof of the mouth with a cautery knife and the 2 large lateral blood vessels

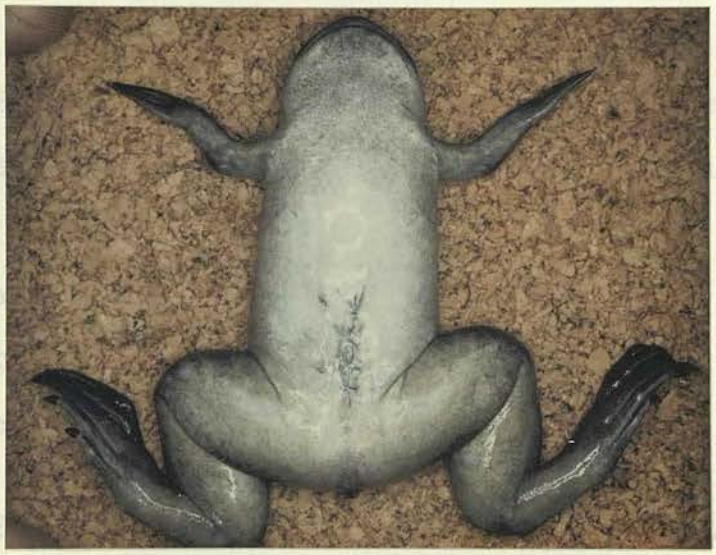

PLATE III.

Healed gonadectomy incision in an adenohypophysectomised Xenopus laevis.

were undercut and pushed laterally to avoid damaging them. The bone was drilled with a dental burr until only a thin layer of cartilage remained intact. This was carefully resected and lifted with a very fine knife leaving the pituitary visible. From this stage the operation site was viewed through a large lens attached to an "anglepoise" type of lamp. The pars distalis was carefully separated from the rest of the pituitary and lifted out using the point of a very fine knife. This method was adapted from that of Hogben (1923), but it was found difficult to ensure removal of the pars distalis alone when suction was used for its removal.

After successful adenohypophysectomy, the melanophores expanded and the toad became dark in appearance (Plate IV).

Recording the extent of gloving in Xenopus laevis.

The nuptial pads or "gloves" of X. laevis are composed of closely set, black-pigmented, hooked spines. Their extent may vary from a small patch on the ventral surface of the 2 middle digits to an area covering the whole ventral surface of the "hand" together with a strip of the ventral surface of the forelimbs up to the axillae (Leslie, 1890; Bles, 1901, 1905; Berk, 1938; Hobson, 1955).

Hobson (1955) described 3 clearly defined stages of gloving. In order to be able to record the mean extent

PLATE IV.

Adenohypophysectomised female Xenopus laevis showing darkening of the skin. (the left side of the toad is damp, the right side is dry).

of gloving in a group of toads, I have given a numerical value to each of Hobson's stages and also to intermediate stages. The numerical value is referred to as the Gloving Index (G.I.) (Table 1 and Plates V-VII). Where, in the text, the term G.I. is related to a group of toads, it indicates the mean Gloving Index.

The observation of the G.I. is partly subjective, and every precaution was taken to ensure consistency in "reading" the G.I. The spines show up best when the toad is immersed in water and the skin is viewed at an acute angle. The G.I. was therefore read with the toad in a jar of water held up at eye level in a well-lit room.

All toads were kept on a light background so that their melanophores were contracted and the gloves could be more easily distinguished.

The preparation of Human Chorionic Gonadotrophin.

The H.C.G. used in these experiments was prepared from the pooled urine of pregnant women. The hormone was extracted by the benzoic acid method of Katzman and Doisy (1932). It was biologically assayed against the First International Standard for H.C.G. using female X. laevis as the test animal (Landgrebe, 1948).

The assay of the gonadotrophic activity of Xenopus partes distales.

Pituitary gonadotrophin activity was assayed by 2 methods; that of Landgrebe, Hobson and Mitchell (1954),

TABLE 1.

The areas of gloving indicated by each value of the gloving index (G.I.)

Value of G.I.	Area of the ventral surface of the fore-legs covered with spines	Hobson's (1955) description	Illustrated in Plate number:-
1	None.	opaque	-
2	Part of 2 inner digits.	-	-
3	First 3 digits.	-	-
4	"Palm" and digits.	hands	V
5	As G.I.4. plus a small area of lower "arm" adjoining the "hand".	-	-
6	As G.I.7 but with the strip incompletely covered.	-	-
7	As G.I.4 plus a wide strip from "hand" to "elbow".	hands-forearm	VI
8	As G.I.7 plus a small area just above the elbow.	-	-
9	As G.I.10 but with the strip on the "upper arm" incompletely covered.	-	-
10	As G.I.7 plus a narrow strip from the "elbow" to the "axilla".	hands-axilla	VII

PLATE V.

A male Xenopus exhibiting the extent of gloving indicated by G.I.4.

PLATE VI.

A male Xenopus exhibiting the extent of gloving indicated by G.I.7.

PLATE VII.

A male Xenopus exhibiting the extent of gloving indicated by G.I.10.

using male X. laevis, and that of Landgrebe (1948), using female X. laevis as the test animal. In both types of assay, weight-matched groups of healthy toads of known sensitivity were used, the males weighing between 30 and 50 g. and the females between 50 and 70 g. Urine from male toads was examined under a binocular microscope, firstly at 4 hours after injection, and, if no spermatozoa were then seen, again at 24 hours. In the female toad assay, the presence or absence of oviposited ova 24 hours after injection was the criterion of response.

The H.C.G. preparation used throughout this investigation contained 70 I.U. per mg.

Estimation of blood-sugar concentration.

Blood sugar levels were estimated by the method of Landgrebe and Munday (1954). The toads were pithed, the heart exposed and the pericardium removed. The ventricle was allowed to hang through a hole in a filter paper, the apex cut and the blood collected in a dry crucible. In this way blood was obtained quickly and without contamination with other body fluids. Blood was taken from the crucible with an accurate 0·1 ml. pipette. Two or three 0·1 ml. samples were usually obtained from each animal. The blood sugar was estimated and the mean value in milligrams per cent recorded. Previous experience had shown that the variation in blood sugar levels was considerably less in "starved" animals than those recently

fed. For this reason, toads used for blood sugar estimations were kept without food for 2 weeks before being anaesthetized.

Thin-layer chromatography.

The methods used were taken from Randerath (1963). Glass plates, 20 cm. x 20 cm. were spread with Silica Gel G. "Merk" using a "Desaga" spreading device. They were dried in air overnight and activated by heating in an oven at 105°C. for 30 min. After spotting, the plates were run by ascending chromatography, the front being stopped 10 cm. from the start.

The following solvent systems were used:-

benzene/methanol (9:1)

cyclohexane/ethyl acetate (1:4)

After running, spots were detected by spraying with concentrated sulphuric acid or antimony trichloride, and examining under an ultra violet lamp, and also by spraying with antimony pentachloride and viewing in visible light.

Extracts of Xenopus ovaries were run in parallel with the following steroids as reference materials:- oestradiol-17β, oestrone, oestriol, progesterone, testosterone and hydrocortisone.

RESULTS.

Investigation of effect of 3 anaesthetics, tricaine methane sulphonate (MS 222, Sandoz), ether, and methane.

Male and female Xenopus adults of approximately the same weight and in groups of 10, were anaesthetized by two methods. The toads were either immersed in an aqueous solution of the anaesthetic, prepared immediately before use, or the solution was injected into the dorsal lymph sac (D.L.S.). Preliminary trials indicated that the following concentrations would be suitable for immersion anaesthesia; tricaine 0·13 per cent, urethane 2·5 per cent and ether 4·0 per cent. 0·01 ml. of a 1·3 per cent solution of tricaine in distilled water was injected with a tuberculin syringe per gram body weight. The dose necessary to produce anaesthesia with urethane was found to be most closely related to body weight by the formula $x = 3 (y + 8)$ where x was the dose in milligrams and y was the weight of the toad in grams. Solutions of urethane were neutralized before injection. Ether was not injected.

Toads were considered to be anaesthetized if no foot retraction reflex occurred when the digits of the hind legs were pinched firmly three times with a pair of dissecting forceps. Two stages of recovery were observed; "partial", when the withdrawal reflex reappeared, and "complete", when toads placed under water, ventral surface uppermost,

righted themselves three times in succession and exhibited normal swimming movements.

Rapid post-operative recovery is advantageous and the time taken by toads to recover in water and in air after being anaesthetized was compared. Partial recovery of half of each group took place in running water. The toads were supported upon a grid and immersed except for their nostrils. The other half of each group begand their recovery in air, being laid upon damp cotton wool in an open dish until partial recovery was attained. They were then placed on the grid in running water until completely recovered. Toads were not returned to the aquarium until they were capable of swimming normally.

To standardize, as far as possible, the conditions under which the experiments were carried out, the water in which the toads were kept for at least an hour before each experiment, the anaesthetic solutions used for immersion, and the water in which the toads were allowed to recover, were maintained at $22^{\circ} \pm 1^{\circ}C$. During partial recovery in air, no attempt was made to accurately control the ambient temperature which fluctuated between 20° and $24^{\circ}C$.

During the experiment in which temperature was the controlled variable, all the toads and containers of anaesthetic solutions were kept in a tank of water maintained at the required temperature to $\pm 0{\cdot}5^{\circ}C$ by a thermostatically controlled water heater and circulator. Twenty-four hours later the toads were anaesthetized, and, for

the duration of the experiment, were kept in beakers of anaesthetic solution in the temperature regulated tank.

Anaesthesia by Immersion.

The times taken for male and female toads to become anaesthetized, and to recover partially and completely after ether, were not affected by the weight of the animal. The figures obtained suggest that partial and complete recovery of the male toad is delayed compared with that of the female after ether treatment. When tricaine and urethane were used, the time taken by toads to become anaesthetized and recover lengthened as their weight increased. Following the use of these two anaesthetics the female toad, in general, recovered more rapidly than the male. The times taken for anaesthetization and recovery were shortest with ether. There was little difference between the time taken to anaesthetize toads with tricaine compared with urethane but toads took longer to recover completely after urethane anaesthesia (Table 2).

It was found that toads immersed in solutions of tricaine or urethane revived more rapidly when recovery took place in water. The time taken to recover completely was increased by 30 per cent when partial recovery occurred in air. Toads recovered equally quickly, after ether anaesthesia, whether this took place in air or water.

TABLE 2.

Time taken for groups, 10 toads per group, of male and female Xenopus to become anaesthetized and recover after immersion in solutions of ether, tricaine and urethane.

Xenopus		4·0 per cent Ether			0·13 per cent Tricaine (MS 222)			2·5 per cent Urethane		
Sex	Group mean weight (g.)	Time (in minutes) taken to attain			Time (in minutes) taken to attain			Time (in minutes) taken to attain		
		Anaesthesia	Recovery		Anaesthesia	Recovery		Anaesthesia	Recovery	
			Partial	Complete		Partial	Complete		Partial	Complete
Male	23·6±2·3*	2·5±0·9	19·9± 7·3	31·6± 5·9	5·4±2·1	15·1± 6·5	25·1±11·3	8·5±1·9	22·1± 7·0	41·3±15·4
	34·8±2·1	2·8±0·6	23·8± 9·2	31·0± 6·9	7·3±2·7	21·4± 1·2	28·5± 8·1	10·7±2·5	21·0± 7·5	50·0±20·8
	50·6±2·5	3·6±0·8	19·8± 7·7	38·5± 4·7	14·7±5·7	40·5±12·2	59·1±25·3	14·2±2·7	41·6±17·2	91·6±20·4
	64·0±2·7	4·0±0·7	18·9± 5·2	37·3± 7·8	16·8±6·8	45·7±11·5	69·5±18·0	17·9±3·9	43·3±17·3	90·3±18·1
Female	23·8±2·3	2·0±0·6	11·4± 4·8	18·5± 3·8	6·0±2·2	21·2±10·8	29·2±10·9	7·0±1·9	13·5± 4·8	31·9± 9·5
	34·1±1·9	3·6±1·5	18·8± 5·1	32·8± 2·5	10·0±3·1	20·6± 3·7	31·4± 2·8	11·6±1·8	24·6± 8·6	53·9±15·8
	52·7±2·7	3·7±0·6	14·9±11·5	28·3± 6·9	14·6±4·4	27·8±10·9	40·0±16·4	13·1±2·6	35·0±11·5	67·2±20·2
	64·4±3·0	3·2±0·5	16·1± 2·4	30·1± 3·8	15·0±3·9	27·3± 9·0	32·9±10·6	16·8±3·1	40·0±13·4	65·6±11·6
	78·5±2·8	3·7±0·9	14·2± 2·0	31·9± 9·4	16·8±6·5	25·9± 6·5	31·8± 8·1	18·9±2·3	41·5± 9·8	80·3±18·6
	92·2±3·7	3·5±0·8	16·6± 6·9	31·8±10·6	20·2±5·9	22·8± 6·8	34·8±11·6	17·6±4·7	36·3±13·8	80·1±30·0

* Each figure is followed by the standard deviation of the mean.

Repeated Anaesthesia.

The tolerance of Xenopus to repeated anaesthesia was tested in the following way. Ten male and 10 female toads, each about 50 g. in weight, were anaesthetized on 6 successive days with tricaine. Four weeks later the same animals were anaesthetized daily for 6 days with urethane and after a further 4 weeks were anaesthetized on 6 successive days with ether.

The time taken by these 20 toads to become anaesthetized and to recover was not altered by this treatment. In the order in which they were used, one anaesthetic did not influence the response of the toads to the next anaesthetic.

Effect of Temperature.

The effect of temperature on the speed and duration of anaesthesia was investigated. Three groups of toads, each containing 3 males and 3 females of similar weight, were anaesthetized by immersion in 4 per cent ether, 0·13 per cent tricaine and 2·5 per cent urethane. One group was used for each anaesthetic. During the first experiment the temperature of the anaesthetic solutions and water in which the toads recovered was kept at 16°C. During the second and third experiments the temperature of all solutions was kept at 21°C and 26°C respectively. Anaesthesia and subsequent recovery occurred more rapidly as the temperature increased (Table 3).

TABLE 3.

Effect of temperature upon the time, in minutes, taken by groups, 3 male and 3 female toads per group, of Xenopus to become anaesthetized and recover.

Anaesthetic	Temperature of anaesthetic solutions								
	16°C			21°C			26°C		
	Anaesthesia	Recovery		Anaesthesia	Recovery		Anaesthesia	Recovery	
		Partial	Complete		Partial	Complete		Partial	Complete
Ether 4 per cent	5·0±1·4*	17·3± 5·9	28·6± 9·8	4·2±1·4	13·1±5·3	26·7±9·8	3·9±0·4	12·8±3·3	26·1±5·5
Tricaine 0·13 per cent	14·9±4·9	24·1± 6·9	44·3±10·5	11·2±2·4	17·0±2·4	26·8±7·0	9·8±2·8	15·5±2·5	24·2±4·5
Urethane 2·5 per cent	19·7±5·7	29·3±10·1	107·2± 8·2	19·6±3·2	30·7±8·2	75·3±5·9	13·1±2·5	22·5±3·2	53·9±8·8

* Each figure is followed by the standard deviation of the mean.

Anaesthesia by Injection.

Forty male Xenopus, weighing between 23g. and 62g., and 60 females, between 24g. and 87g., were injected with a dose of urethane calculated to produce complete anaesthesia and all but 7 toads responded. The reaction of both male and female Xenopus to urethane was unpredictable and the time which elapsed between injection and anaesthesia varied from 3 to 24 min. Partial recovery in air took between 28 and 84 min. and complete recovery between 67 and 126 min. from the time of injection.

One hundred Xenopus were injected with 130µg. of tricaine per gram body weight. Two toads only were not anaesthetized by this dose. The times taken by the groups of male and female Xenopus of varying weight to become anaesthetized, to recover partially and completely are shown in Table 4.

Male and female Xenopus respond similarly to injected tricaine.

Miscellaneous Effects of the Anaesthetics.

Stress. A measure of the metabolic disturbance, due to immersion in ether, or tricaine or urethane, was obtained by estimating the glucose content of the toads' blood at various times after anaesthesia. The blood sugar level of uninjected controls and controls injected with distilled water was also measured. These results were compared with the hyperglycaemia produced in Xenopus after an injection of 5µg. of adrenaline hydrochloride (Table 5).

TABLE 4.

Time taken for groups, 10 toads per group, of male and female Xenopus to become anaesthetized and recover after an injection of 130 µg. of tricaine per gram body weight.

Xenopus		Time, minutes, taken to attain		
Sex	Group mean weight (g.)	Anaesthesia	Recovery	
			Partial	Complete
Male	23·1 ± 2·4*	3·7 ± 1·2	23·9 ± 6·7	37·1 ± 8·4
	34·5 ± 0·6	3·8 ± 1·5	18·9 ± 7·1	33·5 ± 8·4
	50·8 ± 1·5	5·1 ± 3·0	19·3 ± 6·5	31·2 ± 4·9
	62·0 ± 2·7	4·4 ± 1·8	18·3 ± 5·5	27·0 ± 8·1
Female	22·9 ± 1·5	3·2 ± 0·9	18·8 ± 5·1	29·2 ± 5·4
	34·5 ± 1·0	5·7 ± 2·5	20·2 ± 5·2	35·2 ± 7·2
	50·2 ± 2·4	3·4 ± 1·2	23·6 ± 6·0	37·2 ± 5·4
	61·9 ± 3·3	3·4 ± 0·7	19·5 ± 12·8	29·6 ± 10·9
	77·1 ± 3·0	4·6 ± 1·6	18·6 ± 7·4	30·3 ± 8·5
	94·8 ± 3·5	4·2 ± 1·5	20·7 ± 6·2	32·4 ± 7·9

* Each figure is followed by the standard deviation of the mean.

TABLE 5.

Blood sugar levels of Xenopus at various times after anaesthesia with ether, tricaine, urethane and following an injection of 5 µg. adrenaline hydrochloride and distilled water.

Treatment	Number of		Blood sugar mg. per cent.			
	Toads	Estimations	20 min.	60 min.	120 min.	240 min.
Immersion, 4 per cent ether	11	25	54·4	104·3	117·1	118·7
Immersion 0·13 per cent tricaine	11	26	52·5	73·1	91·6	81·5
Immersion 2·5 per cent urethane	10	19	45·0	74·3	80·7	113·6
Injected 5 µg. adrenaline hydrochloride	8	19	64·6	70·7	102·0	126·0
Injected control	4	8				37·5
Uninjected control	6	17	31 mg. per cent.			

The hyperglycaemic action of ether, urethane and to a lesser extent tricaine was similar to that following the injection of adrenaline.

Spermiation. In the concentration used (Table 2), none of the anaesthetics had any gametokinetic activity. It was found that a 5 per cent solution of urethane caused the release of spermatozoa in 1 of the 10 toads in the experiment. Eight per cent ether and 0·26 per cent tricaine did not cause spermiation.

Mucification. When Xenopus were immersed in ether-water mixtures they secreted large quantities of mucus from their cutaneous glands. Immersion in, or injection of, either tricaine or urethane did not cause this secretion.

Although X. laevis were most quickly anaesthetized by immersion in an ether-water mixture, and recovered most quickly from this method of anaesthesia, there was no other advantage to this method. Ether caused severe mucification which made toads difficult to handle. The ether vapour has unpleasant effects on the operator and involves a fire-risk when cautery is used. Urethane has distinct disadvantages; recovery was delayed after anaesthesia by immersion, and the response was difficult to predict when it was given by injection. Furthermore, there is evidence that urethane induces tumours in rats and mice and may be carcinogenic in man (Ball and Cowen, 1959; Revici, 1961). However, after immersion in a solution of tricaine,

anaesthesia occurred in a conveniently short time except when heavy toads were used. Injection of a dose of tricaine related to the weight of the toad induced anaesthesia within 3-6 minutes. Recovery from anaesthesia induced with tricaine was rapid whichever method of administration was used. As estimated by the level of blood-sugar induced, all 3 anaesthetics caused stress, but the maximum level reached was less with tricaine. In other respects there was little difference between the actions of the 3 anaesthetics. In the doses usually employed, no gametokinetic activity was observed, but doubling the concentration of urethane caused spermiation, as did an ether-water mixture containing free ether.

The results obtained suggested that tricaine was the anaesthetic of choice for use in the operations carried out during this investigation.

THE MALE.

The effect of varying the frequency of feeding on the gloving and spermiation response to injected H.C.G.

This experiment was performed to find out if the interval between feeds affects the gonads and secondary sex characters.

Thirty male X. laevis were divided into 3 equal groups of similar weight and G.I.; their claws were clipped for identification. The groups were fed once a week, once every 2 weeks and once every 4 weeks, respectively, for a period of 14 weeks.

A pilot experiment showed that 60 g. liver is more than the amount eaten by 10 males at one feed, so this amount of food was given at each feed. Food was given in the late afternoon and uneaten liver removed on the next morning by straining the water in the tank. It was weighed after drying on the strainer for 1 hour, as it had been shown previously that soaked liver reached its original weight in this time under these conditions.

During the period of the experiment, there was little difference between the amounts of liver eaten by the groups fed weekly and fortnightly, but far less was eaten by the group fed once every 4 weeks (Table 6). This confirms subjective impressions that it is difficult to get a toad to eat when it has been left for long without feeding.

TABLE 6.

The effect of feeding frequency on the amount of liver eaten by male Xenopus laevis.

Date of feeding	Weight of liver eaten (in grams)		
	Toads fed at 1 week intervals	Toads fed at 2 week intervals	Toads fed at 4 week intervals
11/6/59	22·0	23·0	
18/6/59	24·5		
25/6/59	20·0	22·0	
2/7/59	12·0		8·0
9/7/59	12·0	24·0	
16/7/59	9·5		
23/7/59	15·0	18·0	
30/7/59	9·0		11·5
6/8/59	15·0	28·0	
13/8/59	25·0		
20/8/59	7·0	25·0	
27/8/59	9·0		nil
3/9/59	5·0	16·0	
10/9/59	6·0		
Total amount of liver eaten during 14 weeks	191·0	133·0	19·5

The gloving indices of the 3 groups varied little during the 13 weeks (Figure 1). The means of 140 observations for each group were $3{\cdot}9 \pm 0{\cdot}15$, $3{\cdot}9 \pm 0{\cdot}12$ and $4{\cdot}4 \pm 0{\cdot}39$ for the weekly, fortnightly and 4-weekly fed groups respectively.*

Each toad was then injected with H.C.G. to find out if the difference in feeding had affected spermiation and gloving response. Two injections were given, one of 35 I.U. followed after 4 days by one of 70 I.U.

Table 7 shows that the spermiation response of all 3 groups to the 1st injection was similar. The response of the groups to the 2nd injection is not significantly different.

The G.I. of all 30 toads rose to the maximum within 20 days of the 1st injection. There was no real difference between the gloving responses of the 3 groups (Figure 2).

<u>The effect of the environment on the spermiation responses to exogenous gonadotrophin</u>.

Two observations have indicated that environmental changes might affect the release of endogenous gonadotrophin in X. laevis. If male toads are kept in glass jars after injection of gonadotrophin, they continue to shed sperm for some days, whereas, if returned to tanks, they soon cease spermiating. Male toads kept together in a tank, during an assay, appeared to be less sensitive to

* The second of each pair of figures is the Standard Error of the Mean.

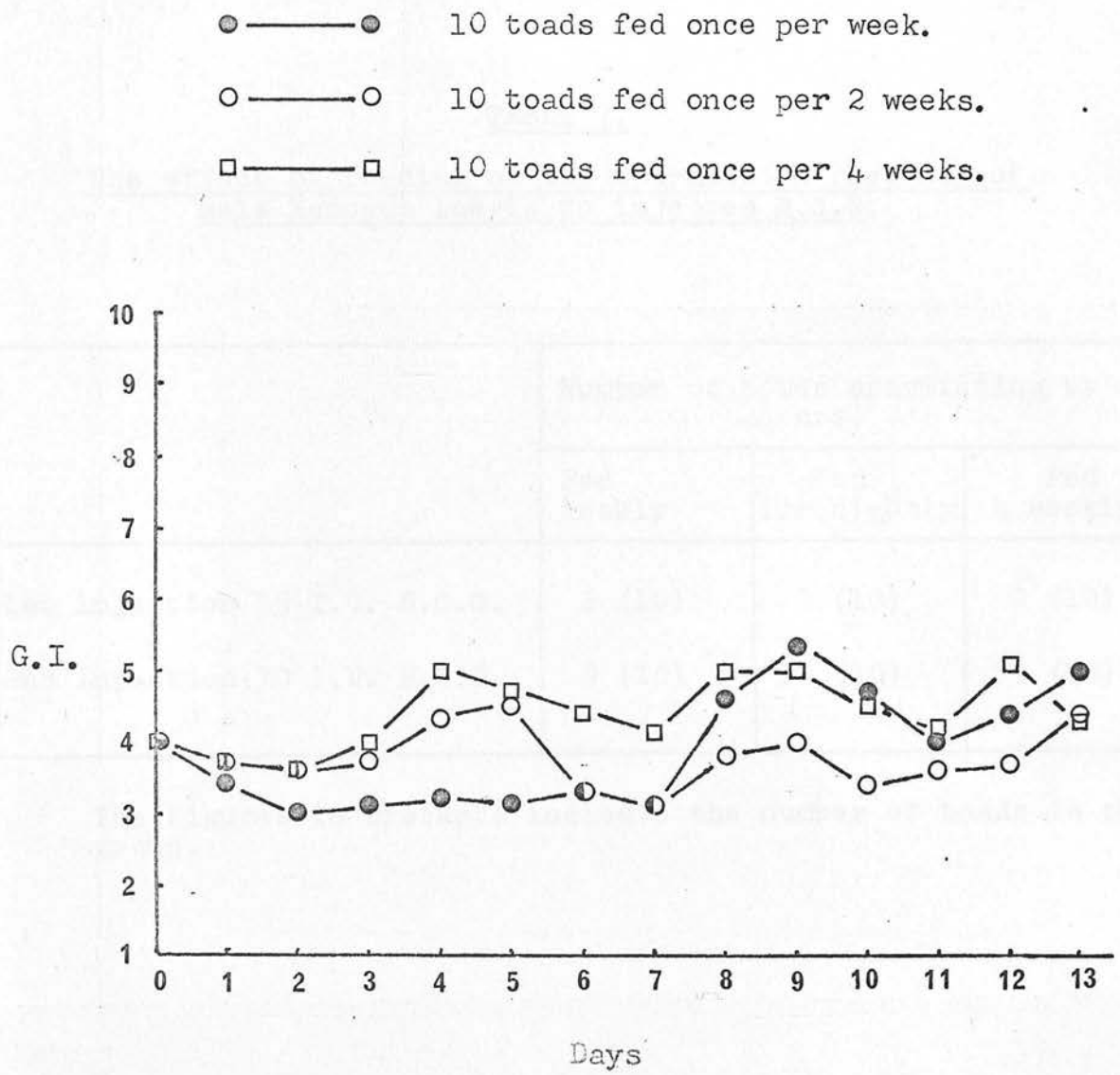

Figure 1.

The effect of feeding on gloving in male Xenopus laevis.

TABLE 7.

The effect of feeding on the spermiation response of male Xenopus laevis to injected H.C.G.

	Number of toads spermiating by 24 hrs.		
	Fed weekly	Fed fortnightly	Fed 4 weekly
1st injection 35 I.U. H.C.G.	2 (10)	3 (10)	2 (10)
2nd injection 70 I.U. H.C.G.	9 (10)	10 (10)	7 (10)

The figures in brackets indicate the number of toads in the group.

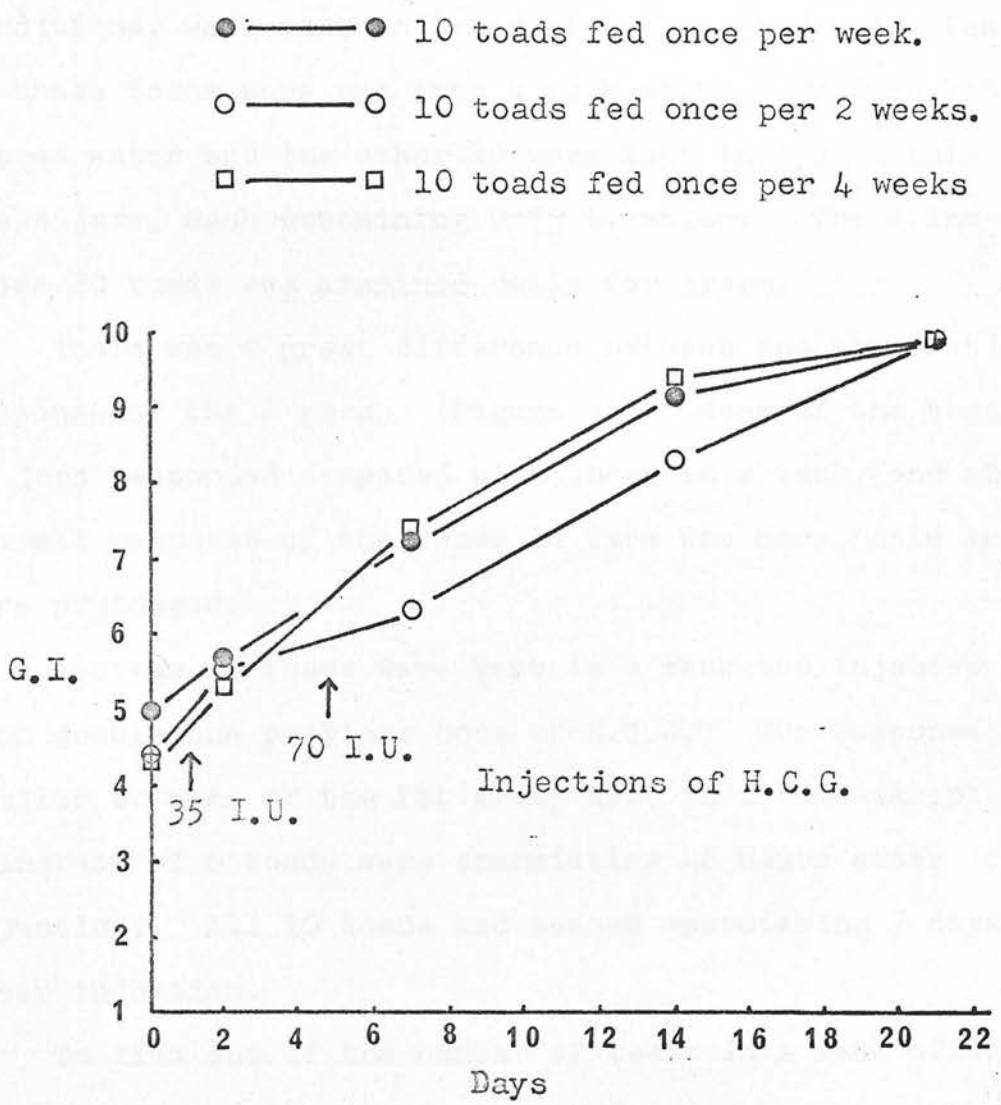

Figure 2.

The effect of feeding on the gloving response to H.C.G.

H.C.G. than similar toads isolated in jars (Hobson, personal communication). The following experiments were performed to investigate this observation.

Twenty male toads, previously kept under identical conditions, were each injected with 20 I.U. H.C.G. Ten of these toads were put into a tank which contained 7·5 litres water and the other 10 were kept in individual glass jars, each containing 0·75 L. water. The urine of these 20 toads was examined daily for sperm.

There was a great difference between the spermiation response of the 2 groups (Figure 3). More of the toads in jars responded compared with those in a tank, and the overall response of the toads in jars was more rapid and more prolonged.

Another 10 toads were kept in a tank and injected with double the previous dose of H.C.G. The response was similar to that of the 1st group kept in a tank except that 9 instead of 6 toads were spermiating 48 hours after injection. All 10 toads had ceased spermiating 7 days after injection.

To find out if the number of toads in a tank affects the individual response to injected gonadotrophins, a group of 5 toads were each injected with 20 I.U. H.C.G. and kept in a tank. The spermiation response was identical to that of the similarly injected group of 10 which was kept in a tank.

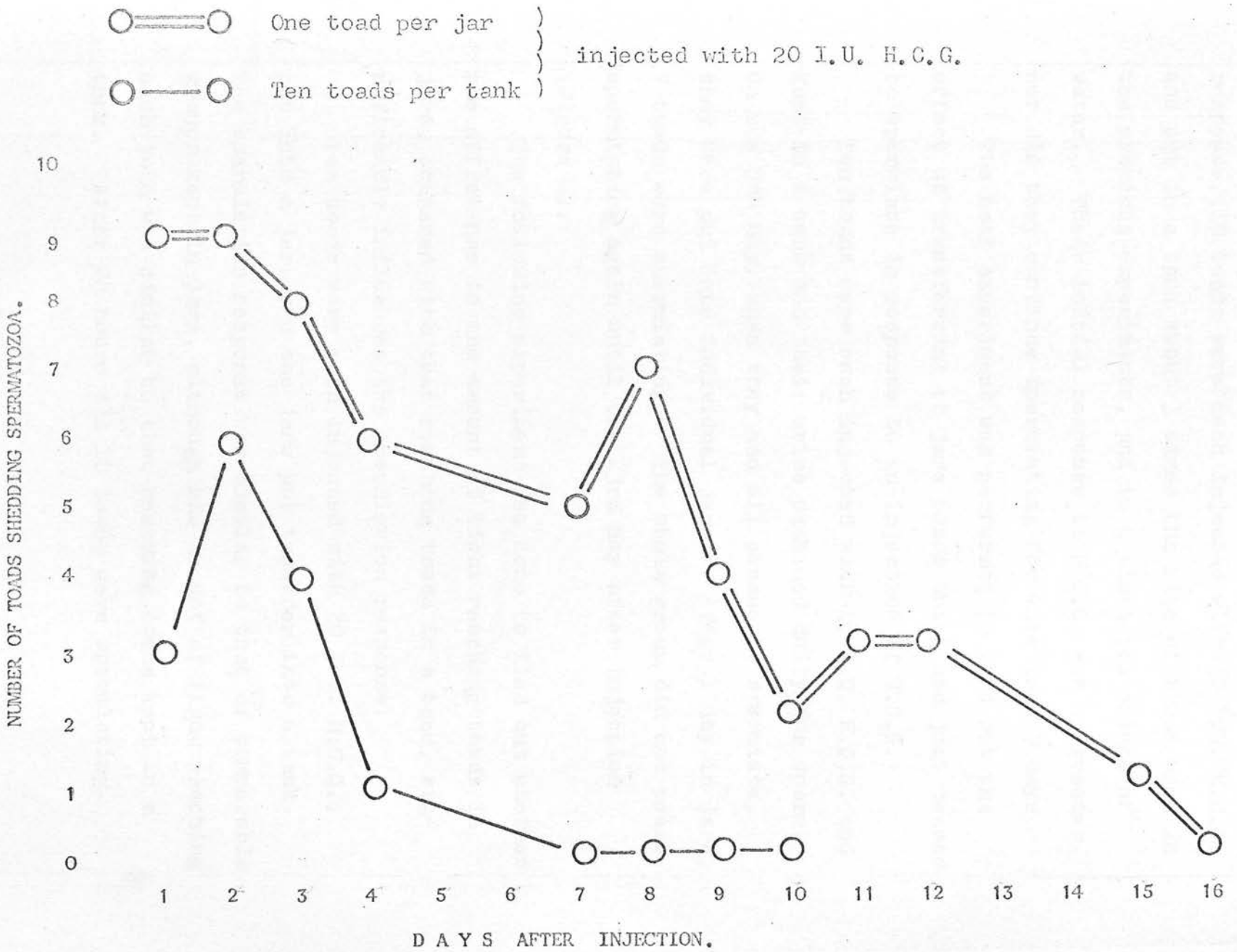

Figure 3.

The spermiation response of male Xenopus laevis kept in jars and in a tank.

To find out if the size of the tank affected the response, 10 toads were each injected with 20 I.U. H.C.G. and put in a tank about 3 times the size of those used in the previous experiments, and in 4 times the volume of water. Their initial response to H.C.G. was no greater, nor did they continue spermiating for more than 9 days.

The next experiment was performed to find out the effect of transferring to jars toads which had just ceased to spermiate in response to an injection of H.C.G.

Ten toads were each injected with 40 I.U. H.C.G. and kept in a tank and their urine examined daily for sperm. On the 8th day, when they had all ceased to spermiate, they were put into individual jars. After 1 day in jars, 7 toads were spermiating. The whole group did not cease spermiating again until the 23rd day after injection (Figure 4).

The following experiment was done to find out whether the difference in the amount of light reaching toads in jars, compared with that reaching toads in a tank, significantly influences the spermiation response.

Ten toads were each injected with 20 I.U. H.C.G., put into a jar, and the jars put together into a tank. The spermiation response was similar to that of comparable groups kept in jars, although the amount of light reaching each toad was similar to that reaching toads kept in a tank. After 24 hours all 10 toads were spermiating.

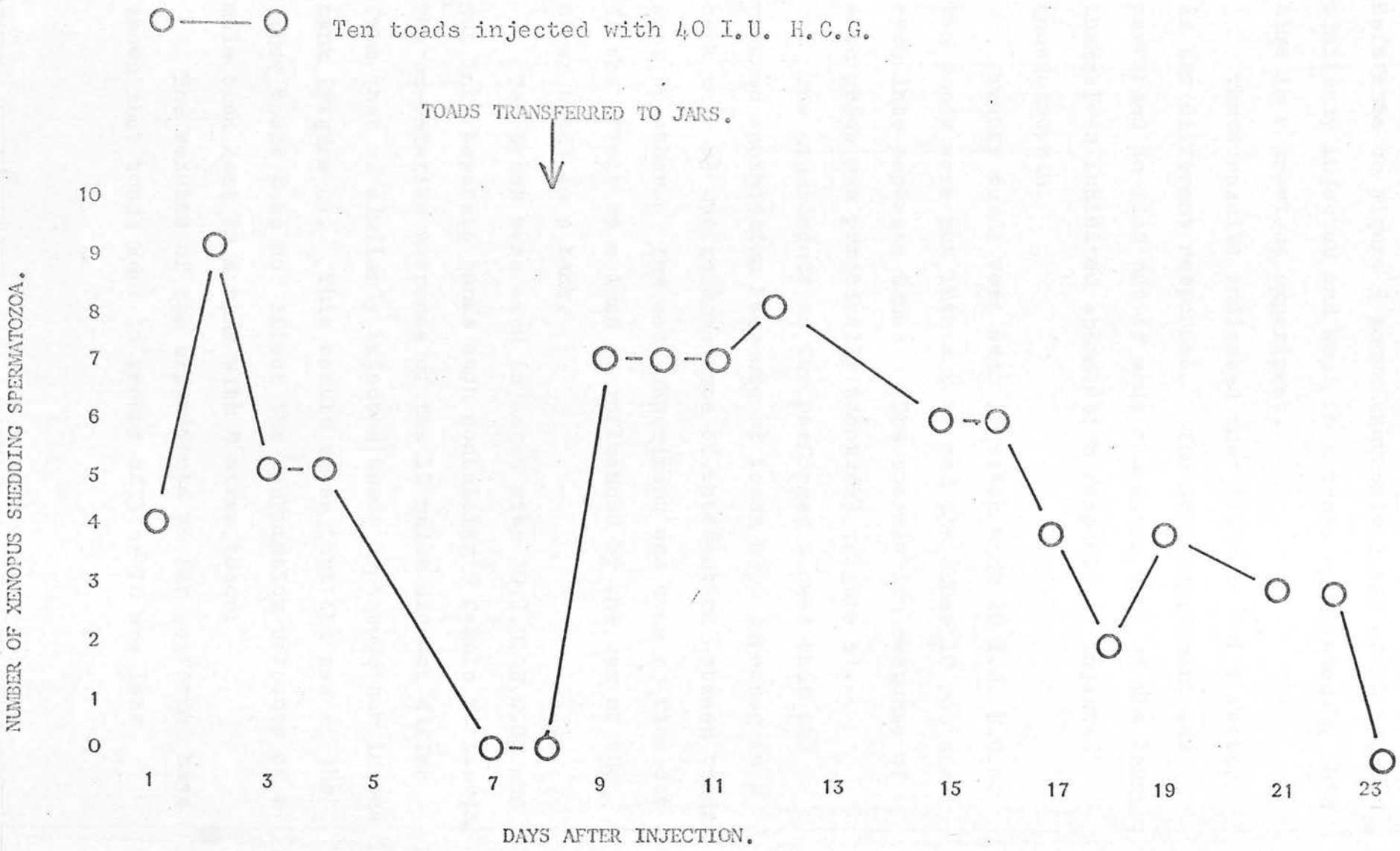

Figure 4.

The effect of isolating male Xenopus laevis after they have ceased spermiating in response to injected H.C.G.

Reference to Figure 3 shows that only 3 out of 10 toads, similarly injected and kept in a tank, spermiated by this time in a previous experiment.

These results indicated that light is not a factor in the different responses. The next experiment was performed to find out if some characteristic of the tanks themselves inhibited spermiation response to injected gonadotrophin.

Twenty toads were each injected with 20 I.U. H.C.G. Ten toads were put into a tank and the other 10 put one each into separate tanks. The spermiation response of each group was practically identical (Figure 5).

The experiments so far performed showed that the reduced spermiation response of toads kept together in a tank must be due to some type of interaction between toads kept together. The next experiment was done to find out if the effect on a toad is influenced by the sex of the other toads in a tank.

Ten toads were each injected with 20 I.U. H.C.G. and put into separate tanks each containing 9 female X. laevis. The spermiation response of the 10 males did not differ from that of similarly injected toads kept together in one tank (Figure 6). This result shows that the sex of the other toads does not affect the spermiation response of a male toad kept in a tank with 9 other toads.

The results of the experiments so far performed have shown that toads kept in groups of 5 or 10 are less

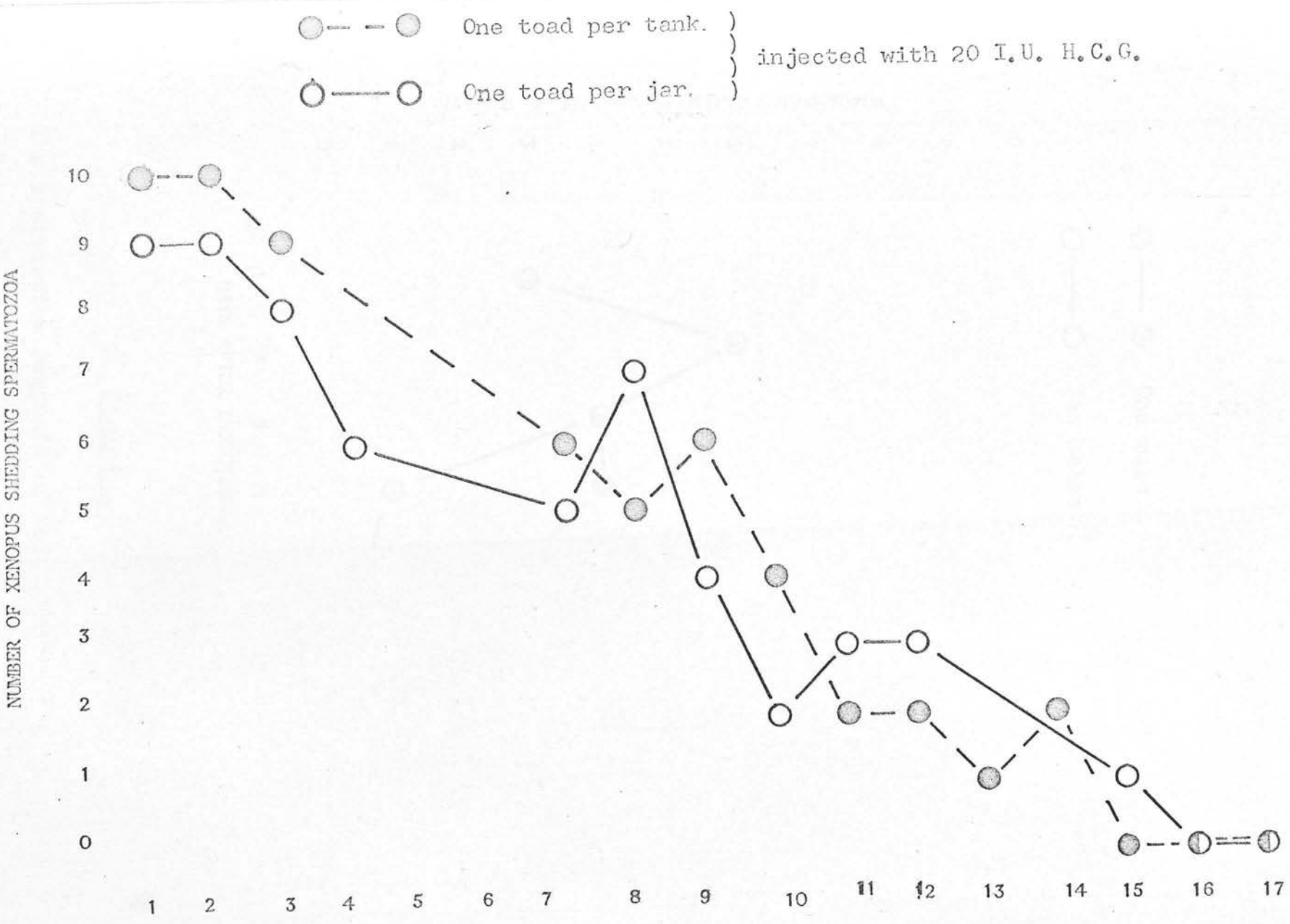

Figure 5.

The spermiation response to injected H.C.G. of male Xenopus laevis isolated in jars and in tanks.

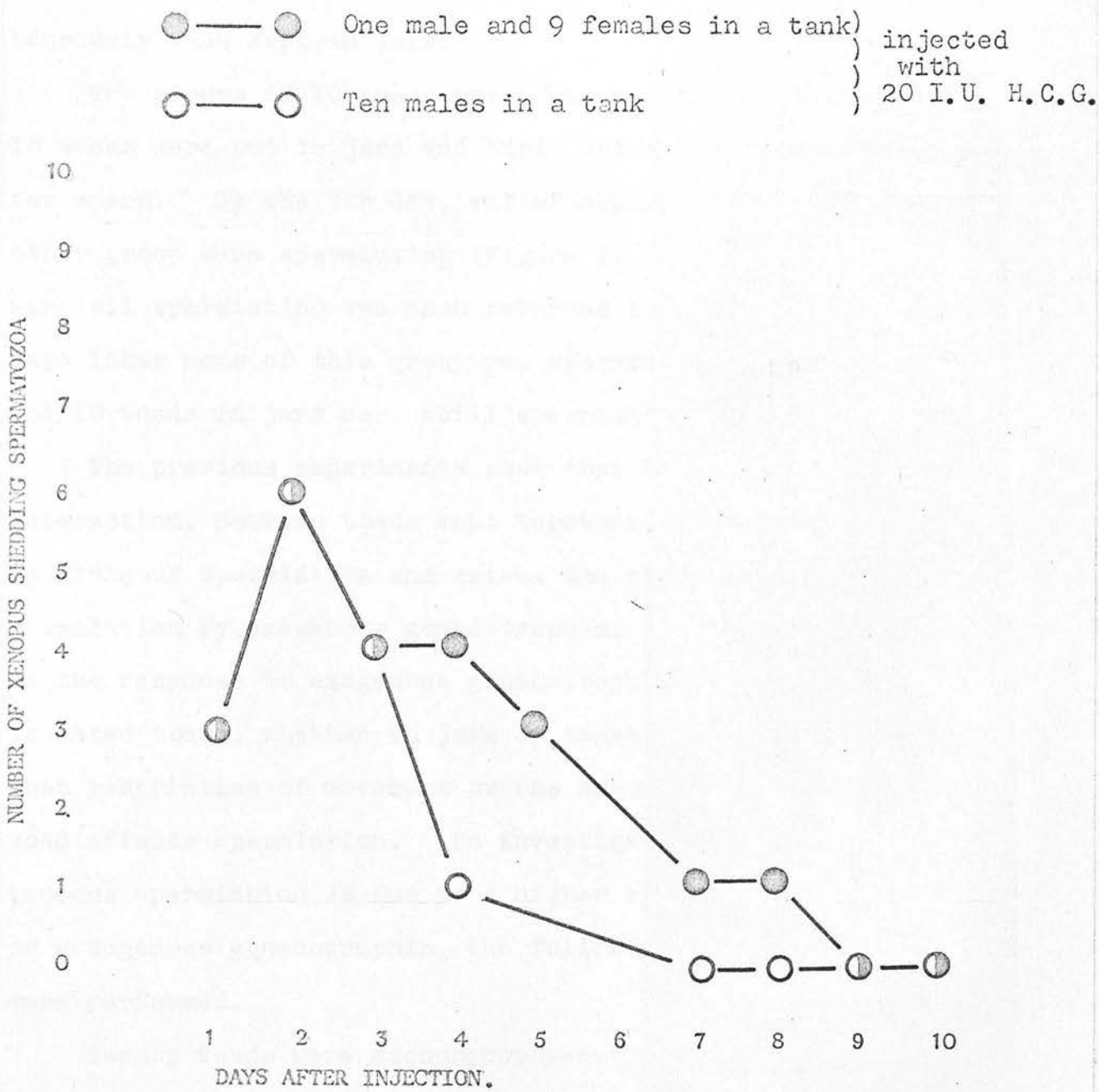

Figure 6.

The spermiation response of male Xenopus laevis kept with males and with females and injected with H.C.G.

sensitive to injected H.C.G. than toads which are isolated, whether in jars or separate tanks. The next experiment was performed to find out if toads will spermiate spontaneously when kept in jars.

Two groups of 10 toads which had not been injected for 18 weeks were put in jars and their urine examined daily for sperm. By the 7th day, all of one group and 9 of the other group were spermiating (Figure 7). The group which were all spermiating was then returned to a tank. Three days later none of this group was spermiating whereas 7 of the 10 toads in jars were still spermiating (Figure 7).

The previous experiments show that there is some interaction, between toads kept together, which inhibits spontaneous spermiation and raises the threshold of stimulation by exogenous gonadotrophin. The similarity in the response to exogenous gonadotrophin between isolated toads, whether in jars or tanks, makes it unlikely that restriction of movement or the amount of water per toad affects spermiation. To investigate whether spontaneous spermiation is due to a higher rate of secretion of endogenous gonadotrophin, the following experiments were performed.

Twenty toads were adenohypophysectomised. Ten were put together into one tank and 10 were put in separate jars. Although more of the toads in jars spermiated (Table 8), there was no significant difference between the 2 groups. On the 6th day after hypophysectomy, when

All toads (last injected 4 months previously) put into jars on day 0.

●– –● Ten toads put into a tank after 6 days.

○——○ Ten toads kept in jars for 16 days.

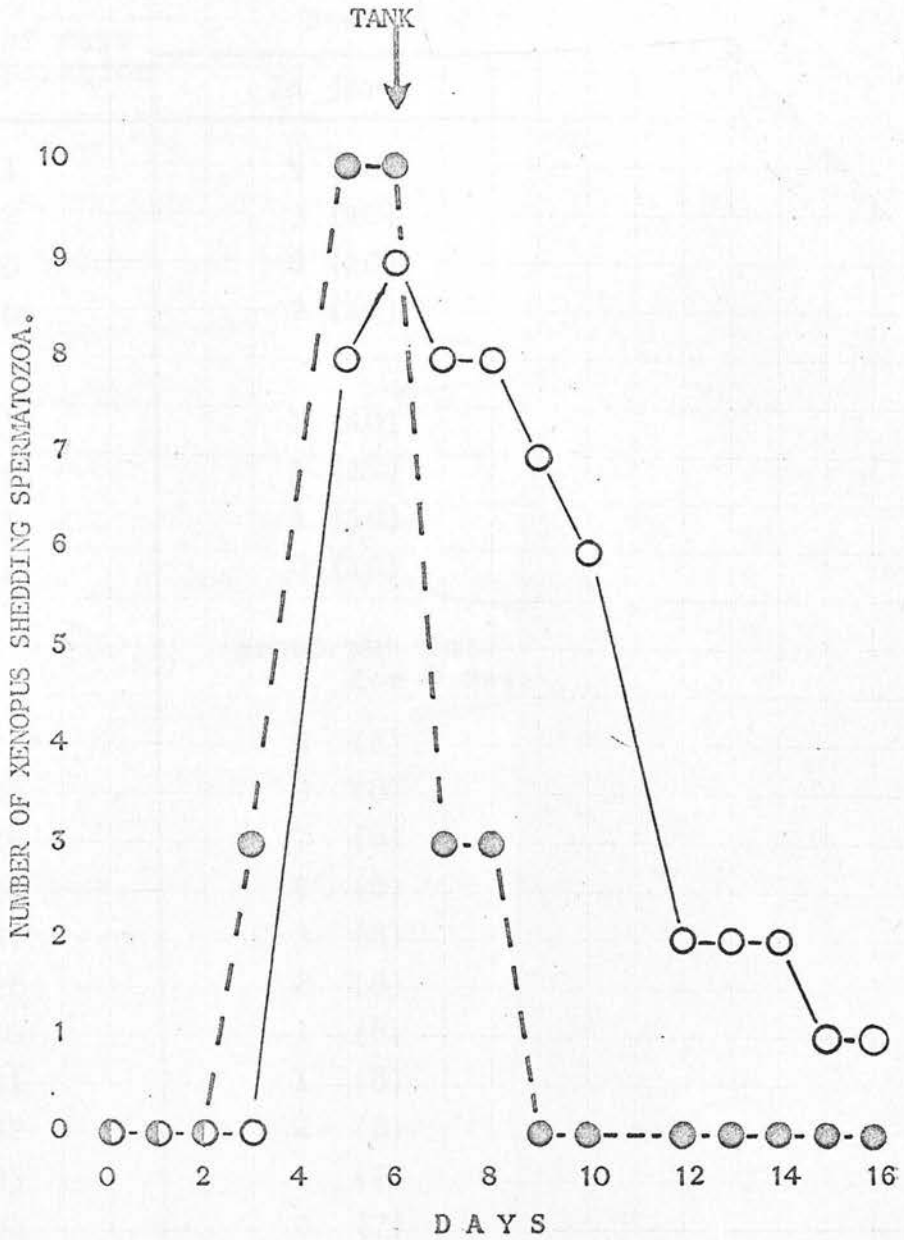

Figure 7.

Spontaneous spermiation of male Xenopus laevis when in jars.

TABLE 8.

Spermiation of adenohypophysectomised male Xenopus laevis kept in jars and in a tank.

Number of days after operation	Number of toads spermiating	
	In jars	In a tank
1	5 (10)	3 (10)
2	3 (10)	0 (10)
3	0 (10)	0 (10)
6	0 (10)	0 (10)
7	1 (10)	0 (10)
8	1 (10)	0 (10)
11	1 (10)	0 (10)
14	0 (10)	0 (10)
15	group put into a tank for 8 days	
23*	0 (8)	0 (9)
24	5 (8)	4 (9)
25	7 (8)	3 (9)
26	5 (8)	4 (9)
27	3 (8)	1 (9)
28	2 (8)	2 (9)
30	1 (8)	1 (9)
31	1 (8)	1 (9)
32	2 (8)	1 (9)
33	1 (7)	1 (9)
34	0 (7)	1 (9)

* each toad was injected with 20 I.U. H.C.G.

The numbers in brackets indicate the number of toads in the group.

spermiation had ceased, the toads in a tank were put into jars and those in jars put into one tank. Although one toad in a jar spermiated, (Table 8), the difference between the groups is not significant. After a further 9 days, each group of 10 toads was put into a tank.

After 8 days in tanks there were 8 and 9 survivors, respectively from the 2 groups of adenohypophysectomised toads. Each toad was injected with 20 I.U. H.C.G. The group of 9 toads was put into a tank and the group of 8 toads put into separate jars. At first, a slightly greater proportion of the toads in jars spermiated (Table 8), but the difference is not significant.

The lack of a significant difference between the spermiation response of hypophysectomised toads in jars and in a tank indicates that the difference between the responses of intact toads is probably due to a difference in endogenous gonadotrophin secretion.

The effect of environment on gloving.

These experiments were performed to find out if the amount of gloving is affected by environment in a similar way to spermiation. The experiments were carried out in a similar way to those on spermiation. Each tank contained 7·5 L. and each jar 0·75 L. water. Jars were arranged in groups so that all toads could see other toads; in this way a difference in visual stimulation was avoided.

To read the G.I. of a toad in a jar, it is not

necessary to handle the toad, whereas with toads in a tank, it is. The first experiment investigated the effect of handling on toads kept under otherwise identical conditions.

Two groups of 10 toads with matched G.I. were kept individually in jars. Their G.I. was recorded on alternate days, when the water was changed, for a period of 20 days. Ten toads (the "handled" group), were deliberately taken out of their jars and held in the hand each time the water was changed. The water in the jars containing the other 10 toads, (the "unhandled" group), was changed very carefully to avoid disturbing each toad. The G.I. of each group rose by the same amount and at the same rate (Figure 8).

As handling was shown by this experiment not to affect the G.I. over 20 days, no attempt to handle each toad equally was made in subsequent experiments.

Two groups of toads were selected with equal G.I. One group was put in a tank; the toads in the other group were put into individual jars. The G.I. of each group was recorded on alternate days. By the 26th day, the G.I. of the group in jars had risen to a constant 7·5, while that of the group in a tank had remained at approx. 4·0 (Figure 9). The toads in a tank were then transferred to jars and vice-versa and the G.I. recorded for a further 26 days. The G.I. of the group put in jars rose to 7·5

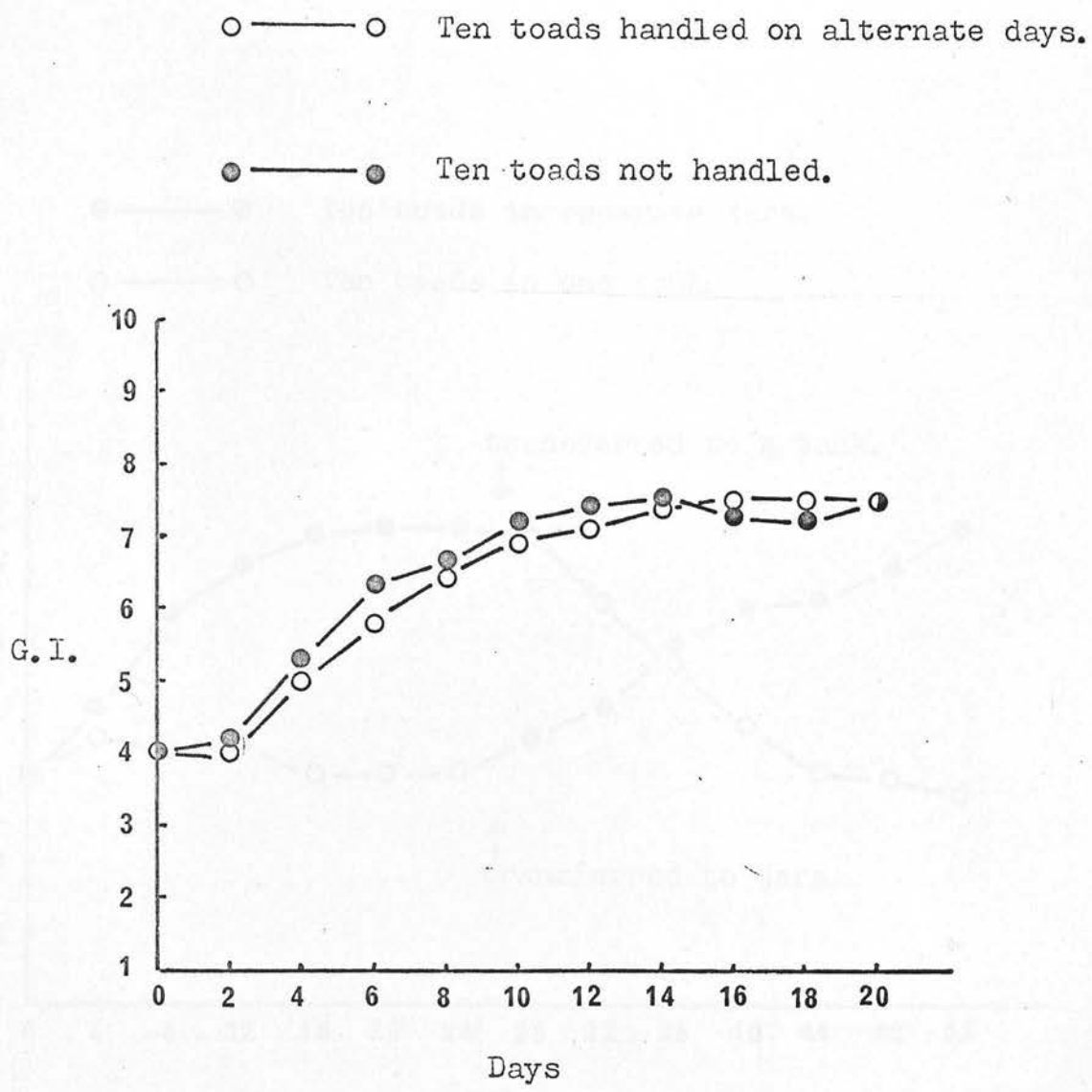

Figure 8.

The effect of handling on gloving in male Xenopus laevis kept in jars.

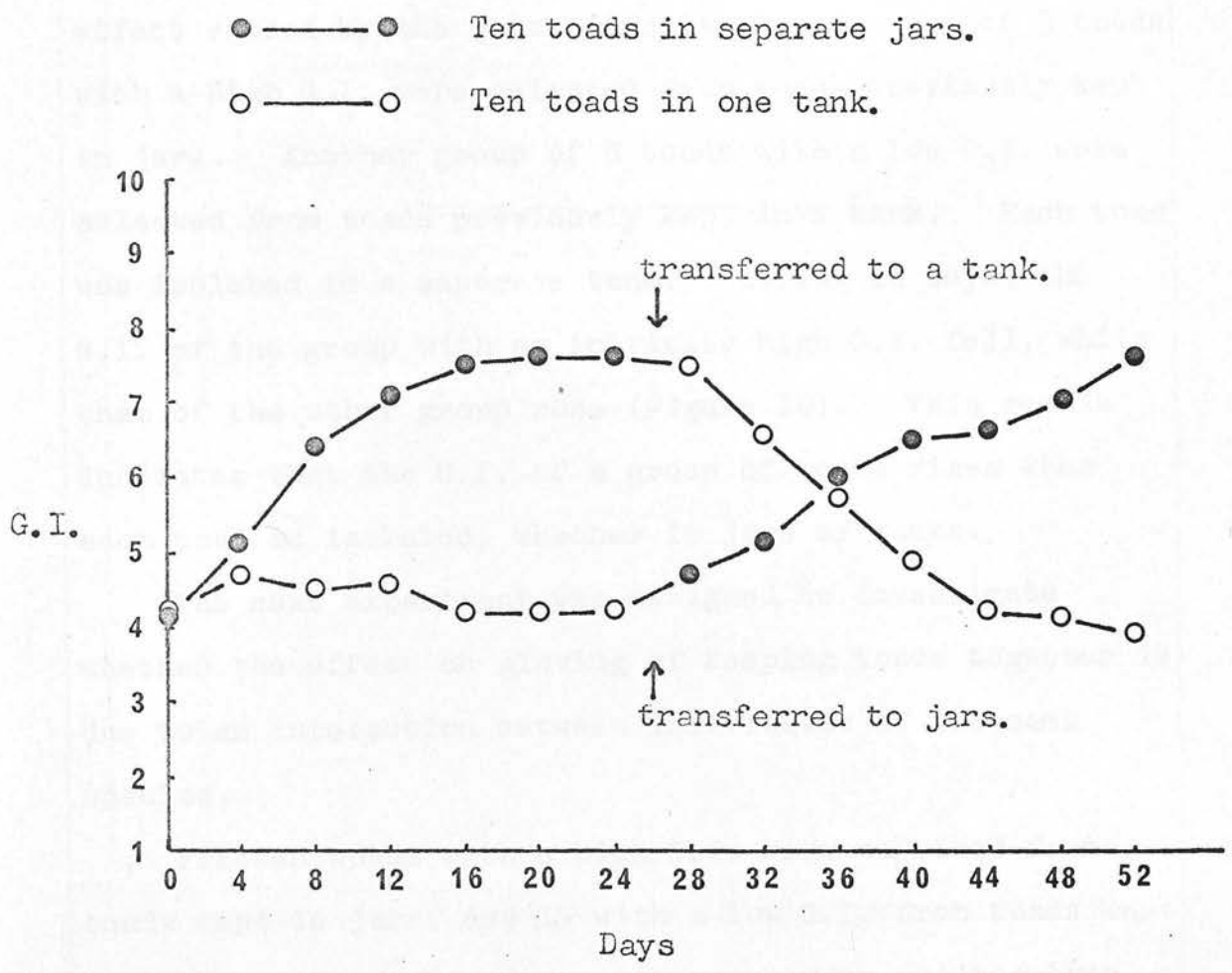

Figure 9.

Gloving in male Xenopus laevis kept together in a tank and isolated in jars.

while that of the group put in a tank fell to 3·8 (Figure 9). This result clearly confirms the previously subjective impression that the extent of gloving is greater in toads kept in jars.

The next experiment was designed to find out whether the G.I. rises as a result of isolation, or because of an effect caused by the jars themselves. A group of 5 toads with a high G.I. were selected from toads previously kept in jars. Another group of 5 toads with a low G.I. were selected from toads previously kept in a tank. Each toad was isolated in a separate tank. During 14 days, the G.I. of the group with an initially high G.I. fell, while that of the other group rose (Figure 10). This result indicates that the G.I. of a group of toads rises when each toad is isolated, whether in jars or tanks.

The next experiment was designed to investigate whether the effect on gloving of keeping toads together is due to an interaction between individuals of the same species.

Fifteen toads with a high G.I. were selected from toads kept in jars, and 15 with a low G.I. from toads kept in tanks. Ten toads from each group were put together into 2 tanks and acted as controls (Figure 11). The other 5 toads from each group were each put into a tank containing 9 goldfish. Goldfish were chosen because the only other genera of completely aquatic Anuran (Pipa pipa

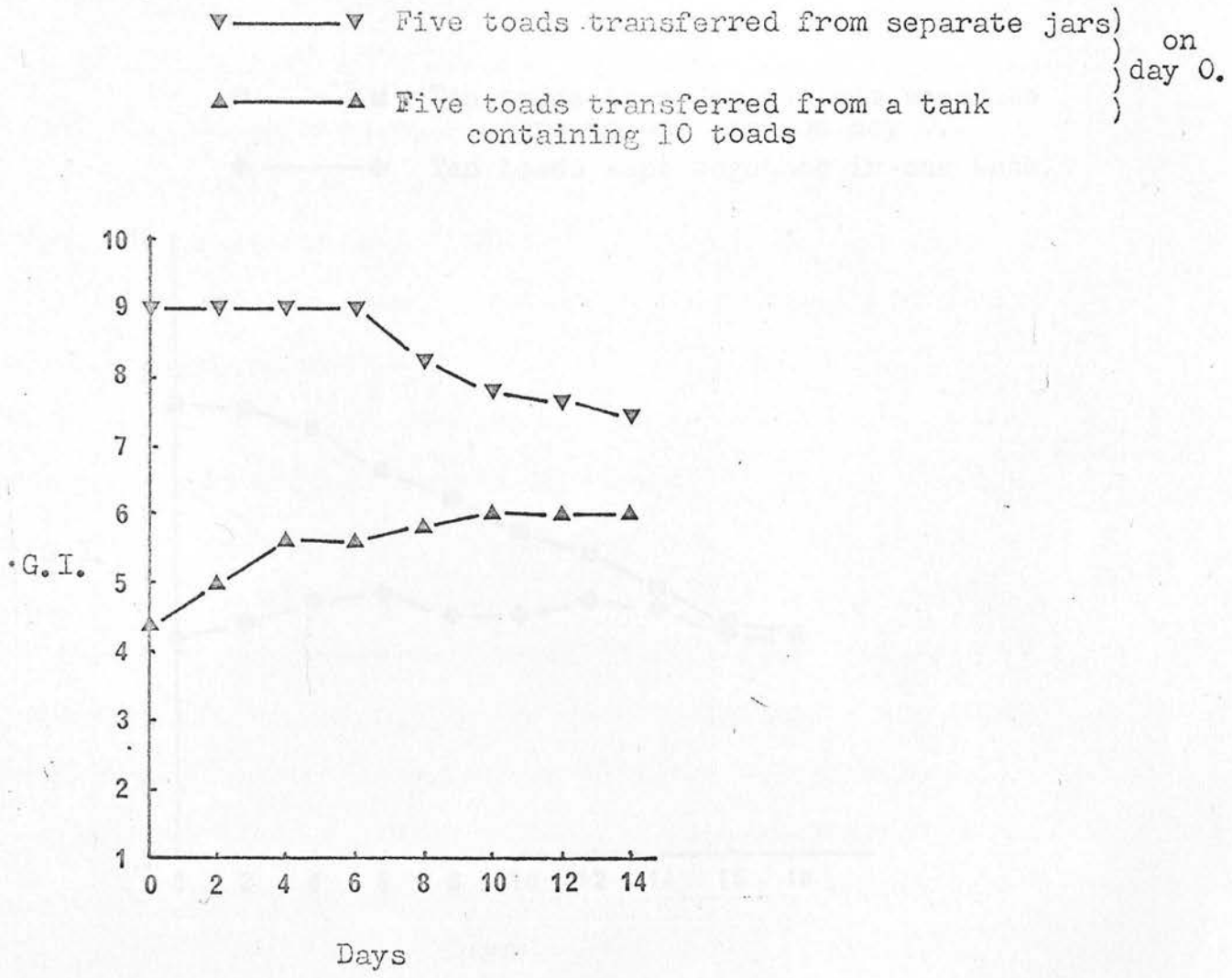

Figure 10.

Gloving in solitary male Xenopus laevis isolated in tanks.

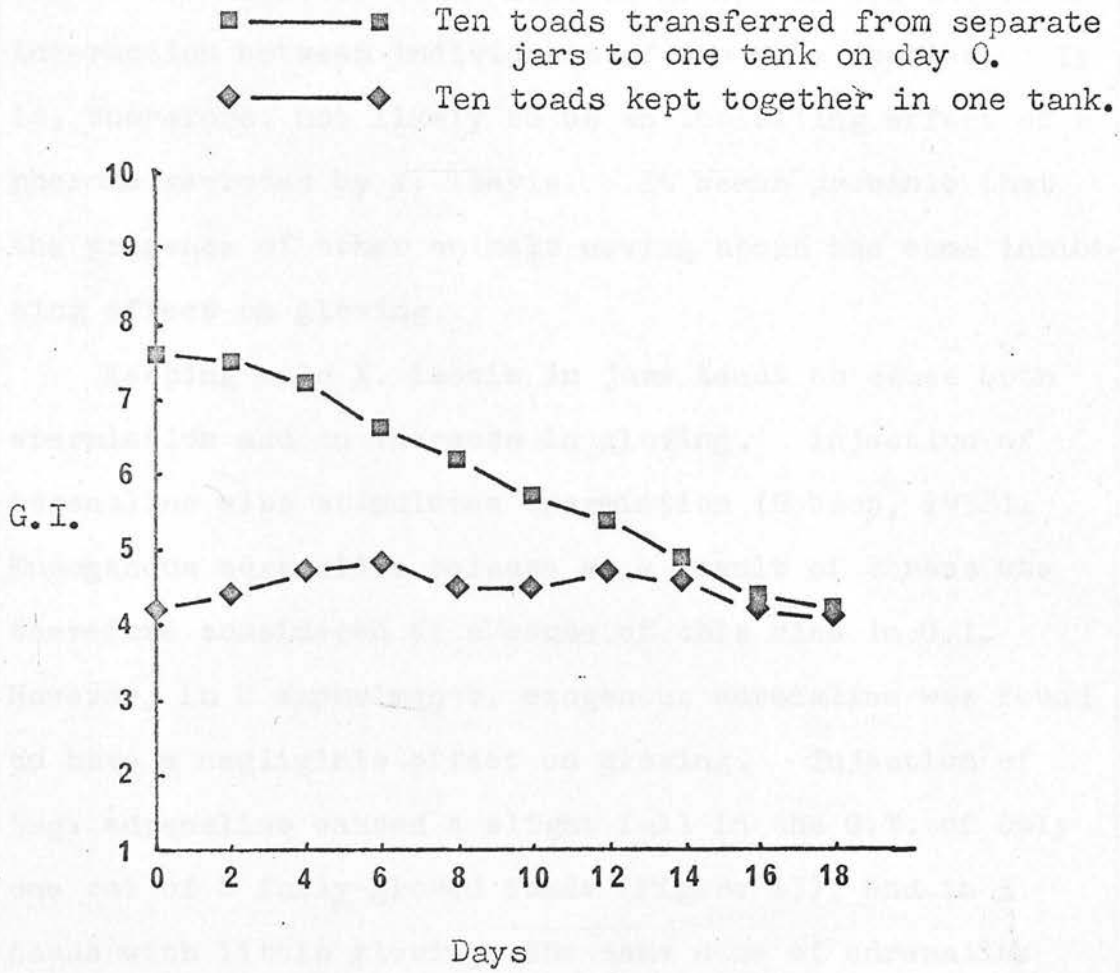

Figure 11.

Gloving in male Xenopus laevis kept together in tanks.

and Hymenochirus) are not available in Europe. It was desirable to avoid the use of closely related species. Figures 11 and 12 show that the effect upon gloving is the same whether a toad is in a tank with 9 other toads or with 9 goldfish.

The result of the last experiment clearly shows that the lowered G.I. of toads kept in groups is not due to an interaction between individuals of the same species. It is, therefore, not likely to be an inhibiting effect of a pherome secreted by X. laevis. It seems probable that the presence of other animals moving about has some inhibiting effect on gloving.

Keeping male X. laevis in jars tends to cause both spermiation and an increase in gloving. Injection of adrenaline also stimulates spermiation (Hobson, 1952). Endogenous adrenaline release as a result of stress was therefore considered as a cause of this rise in G.I. However, in 2 experiments, exogenous adrenaline was found to have a negligible effect on gloving. Injection of 5µg. adrenaline caused a slight fall in the G.I. of only one out of 2 fully-gloved toads (Figure 13), and in 5 toads with little gloving, the same dose of adrenaline caused the same slight rise in G.I. as an injection of distilled water. Injection of 5µg. adrenaline into Xenopus causes the fasting blood sugar concentration to rise to 120 mg.%; this level is not reached in toads deliberately stressed by harrying them for 10 minutes, so

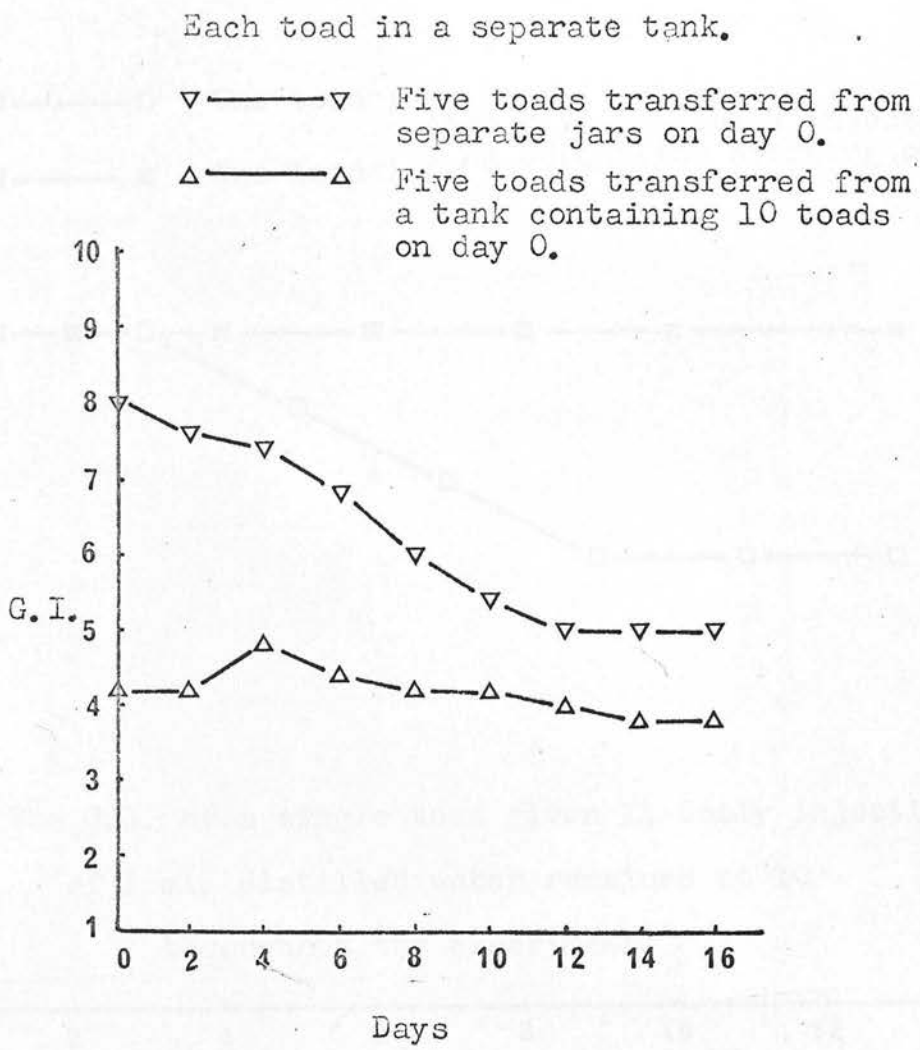

Figure 12.

Gloving in male Xenopus laevis kept in a tank with nine goldfish.

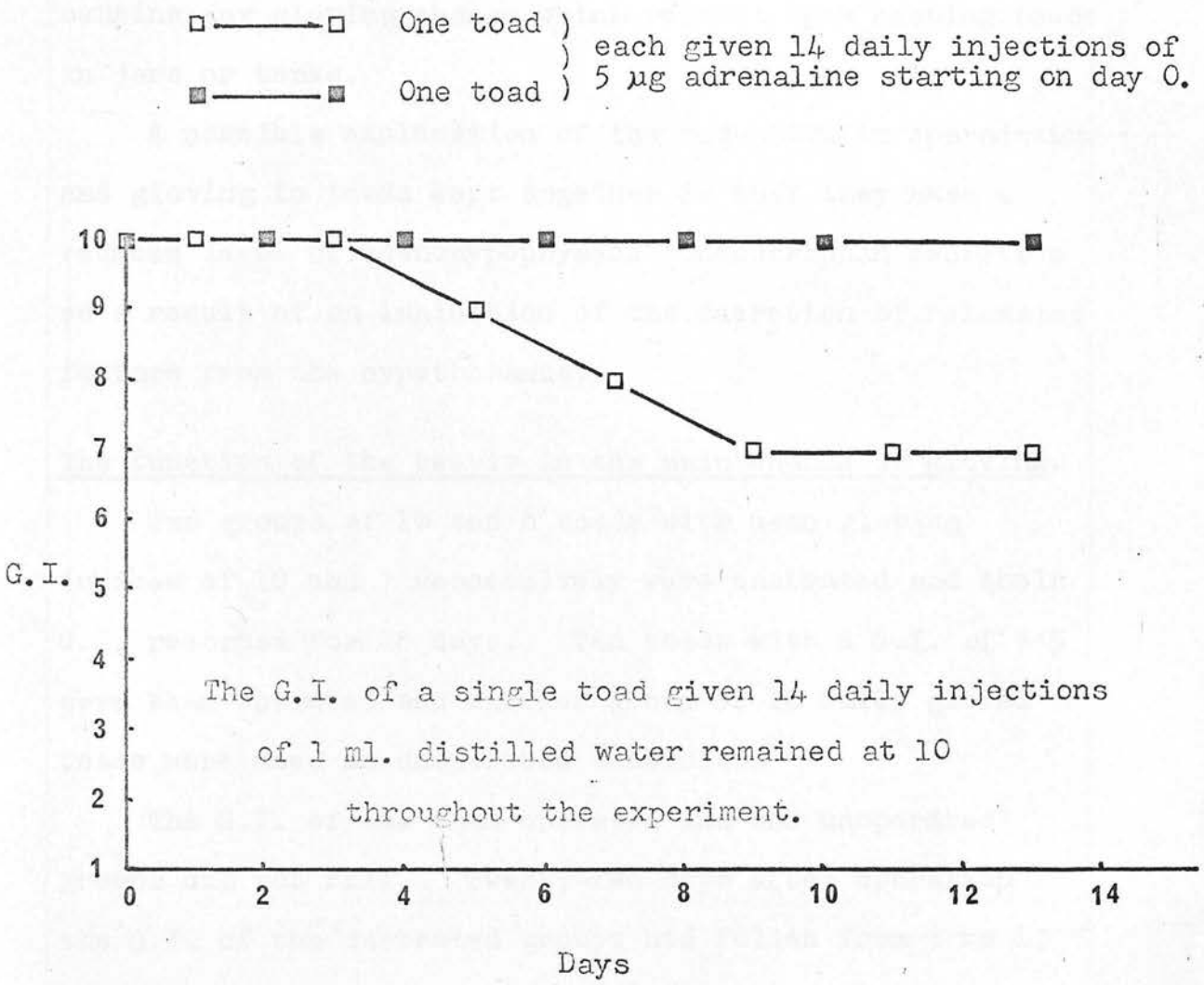

Figure 13.

The gloving response of fully-gloved male Xenopus laevis to injections of adrenaline.

it may be concluded that the amount of endogenous adrenaline released as a result of severe stress is not equivalent to an injection of 5µg. adrenaline. It is, therefore, very unlikely that stress is a factor in causing any gloving change which results from keeping toads in jars or tanks.

A possible explanation of the reduction in spermiation and gloving in toads kept together is that they have a reduced level of adenohypophyseal gonadotrophin secretion as a result of an inhibition of the secretion of releasing factors from the hypothalamus.

The function of the testis in the maintenance of gloving.

Two groups of 10 and 8 toads with mean gloving indices of 10 and 7 respectively were castrated and their G.I. recorded for 26 days. Ten toads with a G.I. of 7·5 were sham-operated and another group of 10 fully gloved toads were used as unoperated controls.

The G.I. of the sham-operated and the unoperated groups did not fall. Twenty-two days after operation the G.I. of the castrated groups had fallen from 7 to 1, and from 10 to 3 respectively (Figure 14). The latter group was examined after a further 2 months and found to have a G.I. of just over 3. They were then killed and examined for the presence of residual testicular tissue; none was found.

These results show that gloving is maintained by the

■—■ Ten unoperated (intact) toads.

□—□ Ten toads castrated on day 0.

▼—▼ Ten toads sham-castrated on day 0.

▽—▽ Eight toads castrated on day 0.

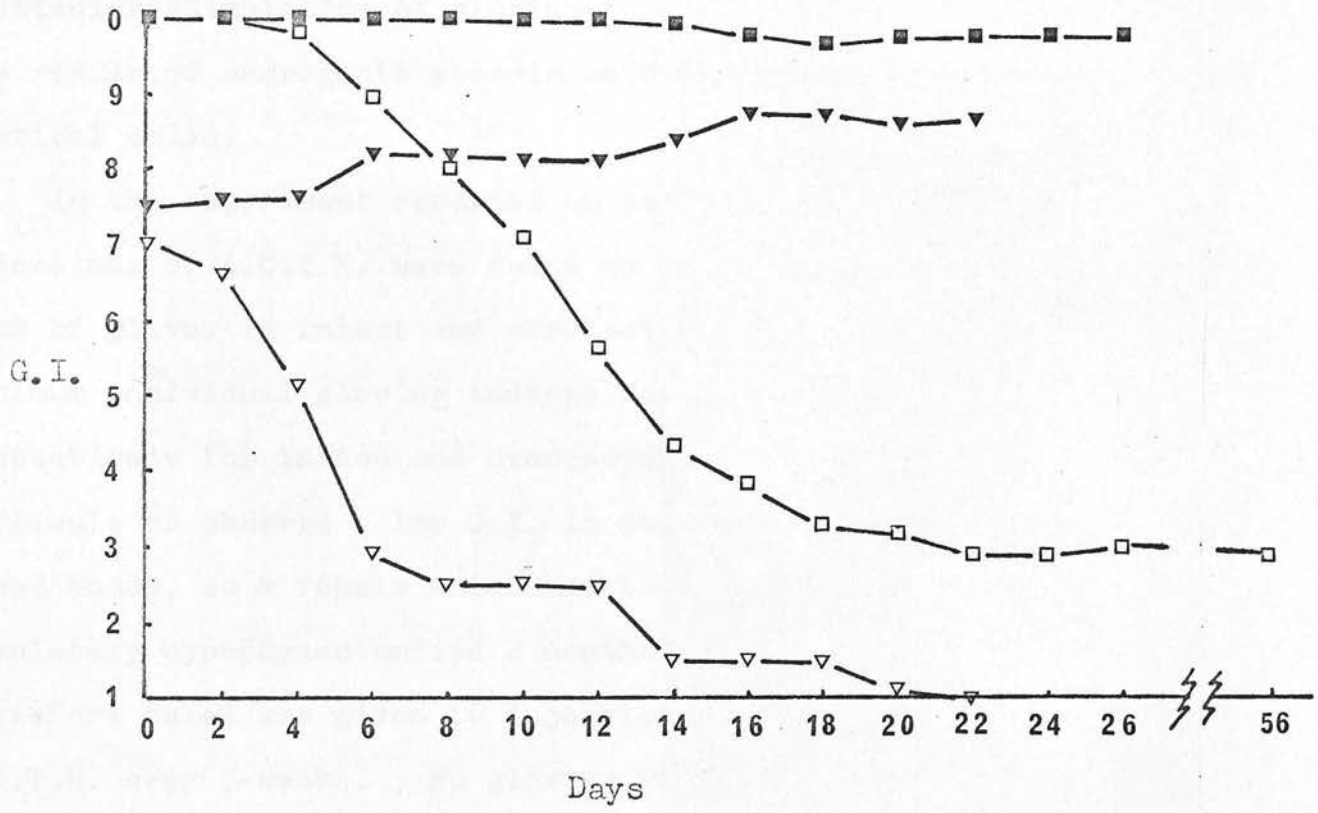

Figure 14.

The gloving response of male Xenopus laevis to castration.

testes, but that the adrenal cortical tissue is probably also concerned in the normal maintenance of gloving. The different levels to which the G.I. of the 2 castrated groups fell indicates different levels of androgenic activity of this organ.

Over 200 stock female Xenopus were examined for gloving. About 30% were found to have some gloving; the G.I. ranged from 2 to 4. It is postulated that the extra-testicular stimulation of gloving in males and females is the result of androgenic steroid secreted by the adrenal cortical cells.

In the experiment reported on pages 154 and 159, injections of A.C.T.H. were found to cause slight development of gloves in intact and ovariectomised females; the maximum individual gloving indices being 3 and 2·5 respectively for intact and ovariectomised toads. It is difficult to observe a low G.I. in dark adenohypophysectomised toads, so a female which had been inadvertently completely hypophysectomised 2 months previously (and was therefore pale) was given 10 injections, each of 30 I.U., A.C.T.H. over 5 weeks. No gloving resulted. The stimulation of gloving in ovariectomised females indicates an extraovarian source of a steroid secreted under corticotrophic stimulation, and supports the hypothesis that this steroid is secreted by the adrenal cortical tissue. The lack of gloving in the hypophysectomised toad is probably

explained by an insensitivity of the adrenal tissue to A.C.T.H. stimulation. The adrenal tissue of Amphibia regresses following hypophysectomy (Chester Jones, 1956).

The effect of exogenous androgen on gloving.

The following experiments were performed to find out whether injections of testosterone will maintain the gloves of castrated toads.

Three fully-gloved males were castrated; 2 of them were given 3 injections of 5 mg. methyl testosterone during the next 6 days and the other acted as an uninjected control. The G.I. of the injected toads remained at 10 for 20 days during which period that of the control fell to 5 (Figure 15). Six weeks after castration the G.I. of the uninjected control toad had fallen to 4; it was then given 4 injections of 5 mg. methyl testosterone over the next 12 days. The G.I. of this toad rose to 10 by the 18th day from its 1st injection (Figure 15).

In a further experiment, 3 ungloved male X. laevis, castrated not less than 7 months previously, were injected with methyl testosterone. This was given in 8 injections over 22 days, and the individual toads received a total of 9, 18 and 36 mg. respectively. Each toad was fully-gloved by the 25th day after the 1st injection, clearly indicating that castration of 7 months duration does not abolish the sensitivity to testosterone (Figure 16).

The following experiments were performed to find out

○—○ Two toads castrated on day 0 and injected with 5 mg. methyl testosterone.

●—● One toad castrated on day 0 and not injected.

□═□ Two toads, castrated 3 weeks previously, injected with 5 mg. methyl testosterone.

▼ ▼ ▼ days injected. (5 mg. per injection)

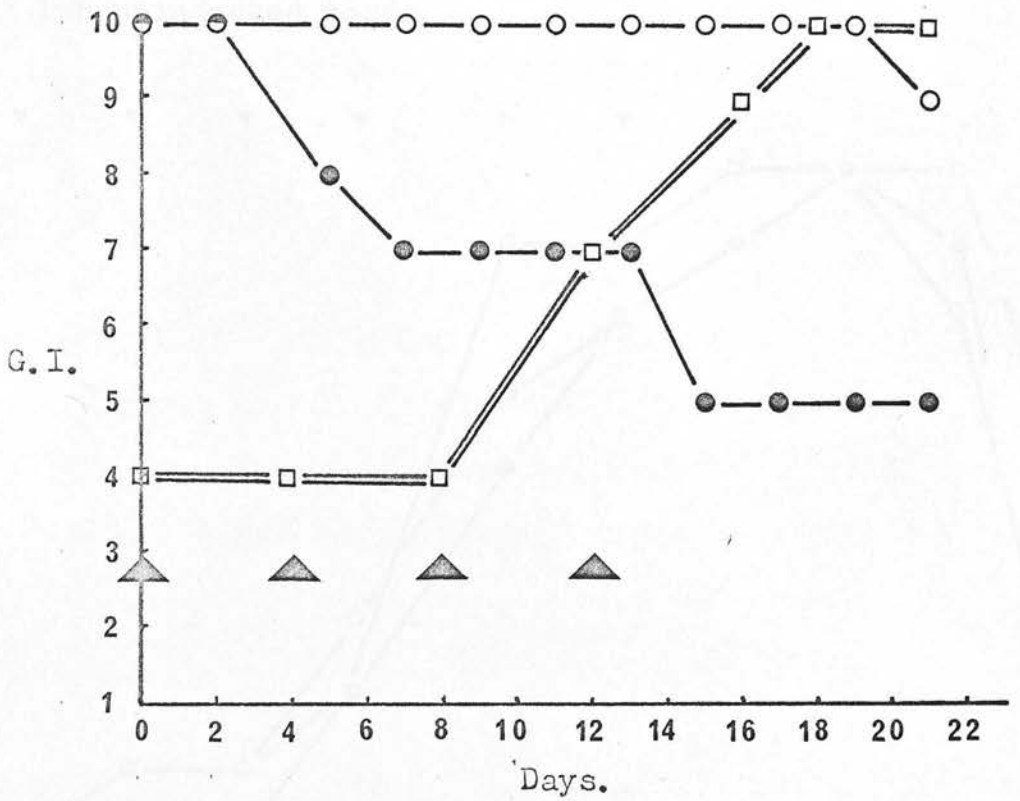

Figure 15.

The gloving response of castrated male Xenopus laevis to injections of methyl testosterone.

▼ Days injected

□—□ One toad injected with a total of 36 mg. methyl testosterone.

○—○ One toad injected with a total of 18 mg. methyl testosterone.

●—● One toad injected with a total of 9 mg. methyl testosterone.

■—■ Two uninjected toads.

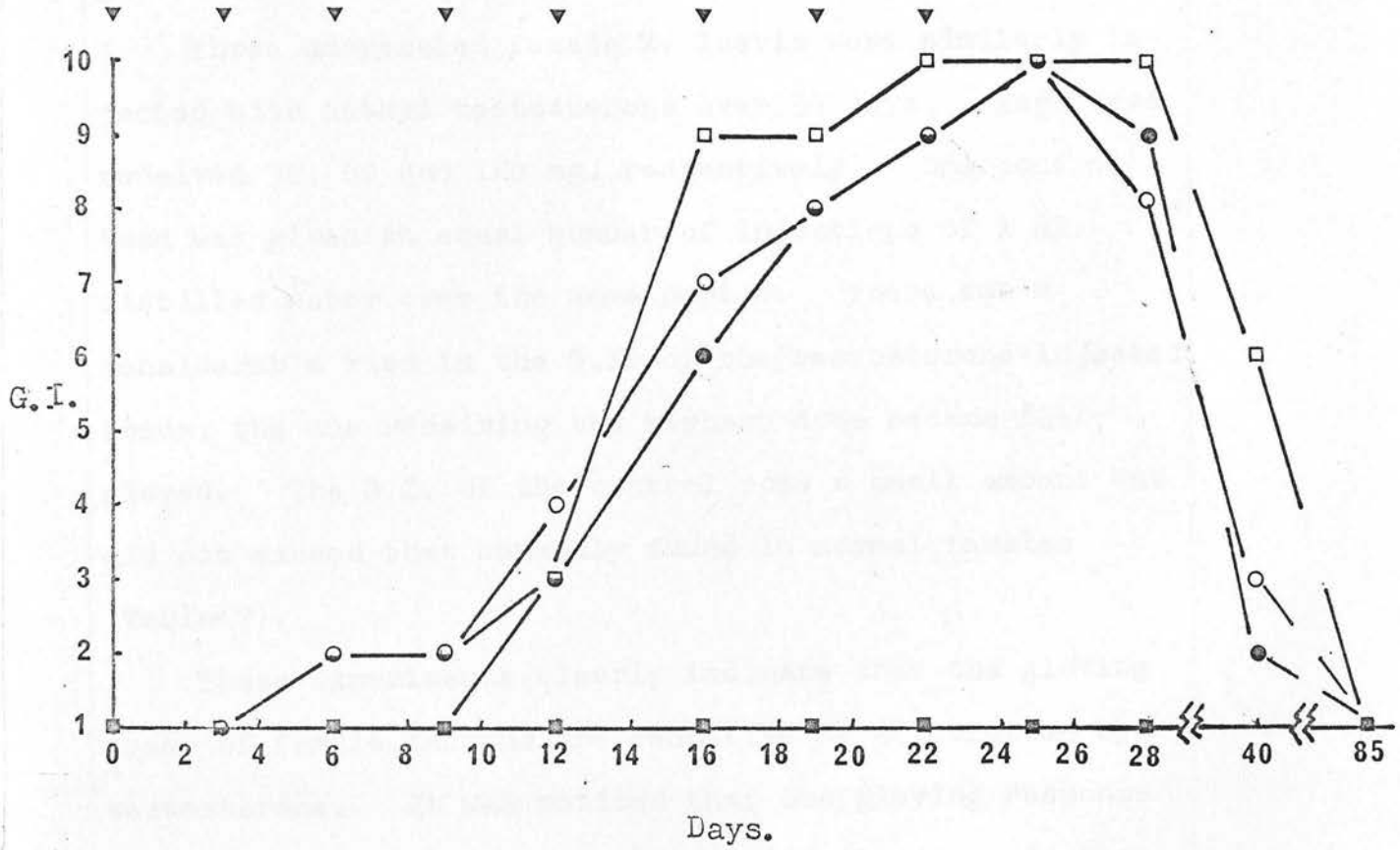

Figure 16.

The gloving response of male Xenopus laevis to methyl testosterone injected 7 months after castration.

if gloving is stimulated in females by exogenous testosterone.

Four female X. laevis were castrated and injected with methyl testosterone over 34 days. One toad received a total of 30 mg., 2 received 60 mg. and one received 84 mg. (Injection of the last toad ceased on the 22nd day when fluid was found to have accumulated in its lymph sacs.) The G.I. of all toads rose; the toad which received the highest dose became almost fully gloved (Table 9).

Three unoperated female X. laevis were similarly injected with methyl testosterone over 34 days. Each toad received 30, 60 and 120 mg. respectively. One control toad was given an equal number of injections of 1 ml. distilled water over the same period. There was a considerable rise in the G.I. of the testosterone-injected toads, the one receiving the highest dose became fully gloved. The G.I. of the control rose a small amount but did not exceed that commonly found in normal females (Table 9).

These experiments clearly indicate that the gloving areas of female Xenopus are sensitive to stimulation by testosterone. It was noticed that the gloving response was no greater and no more rapid, dose for dose, in the ovariectomised females than in the intact female toads. This seems to indicate that ovarian oestrogens do not

TABLE 9

The gloving response of intact and ovariectomised female Xenopus laevis to injected methyl testosterone

Days injected	Day No.	G.I. of toads injected with:							
		1 ml. aq.dest.	3 mg.		6 mg.			12 mg.	
		Intact	Intact	Ovariectomised	Intact	Ovariectomised		Intact	Ovariectomised
→	0	1	3	2	2	3	1	3	4
→	3	1	4	1	3	3	1	3	4
→	7	1	4	3	3	5	2	4	4
→	11	3	5	4	3	5	3	4	6
→	14	3	5	4	5	5	5	5	6
→	18	2	6	6	7	6	7	6	7
→	22	2	7	7	7	6	8	7	8 *
→	26	3	7	7	8	6	8	7	9
→	30	2	7	7	8	7	9	8	8
→	34	2	7	7	9	7	9	10	7
	38	3	7	7	9	7	10	10	6
	47	2	7	7	10	7	9	10	5
	59	2	7	7	10	7	4	5	5

* Last injection of this toad.

significantly antagonise the action of exogenous testosterone. Oestradiol has been found to inhibit gloving in the intact male (Figure 19).

In the following experiment, the efficacy of 3 other steroids was compared with that of testosterone. Each female toad received a total dose of 15 mg. steroid, given in 3 injections over 12 days. Groups of 5 female toads were injected with progesterone, hydrocortisone and cortisone; groups of 2 toads received oestradiol and methyl testosterone respectively. Five control toads were given an equal number of injections of 1 ml. distilled water. The G.I. rose significantly only in the group receiving methyl testosterone (Figure 17). The slight rise in the G.I. of the toads injected with progesterone suggested the trial of this steroid in the intact male.

Three male toads were each given 6 injections of progesterone over 18 days; the total dose received by each toad was 8 mg. A single control toad received 6 injections of 1 ml. distilled water. Figure 18 shows that the G.I. of the toads receiving progesterone rose no higher than the toad receiving distilled water. The fall of the G.I. to 2 by the 43rd day after the 1st injection of progesterone, in each toad surviving until then, suggests a possible negative feed-back effect on the toads' own pituitaries.

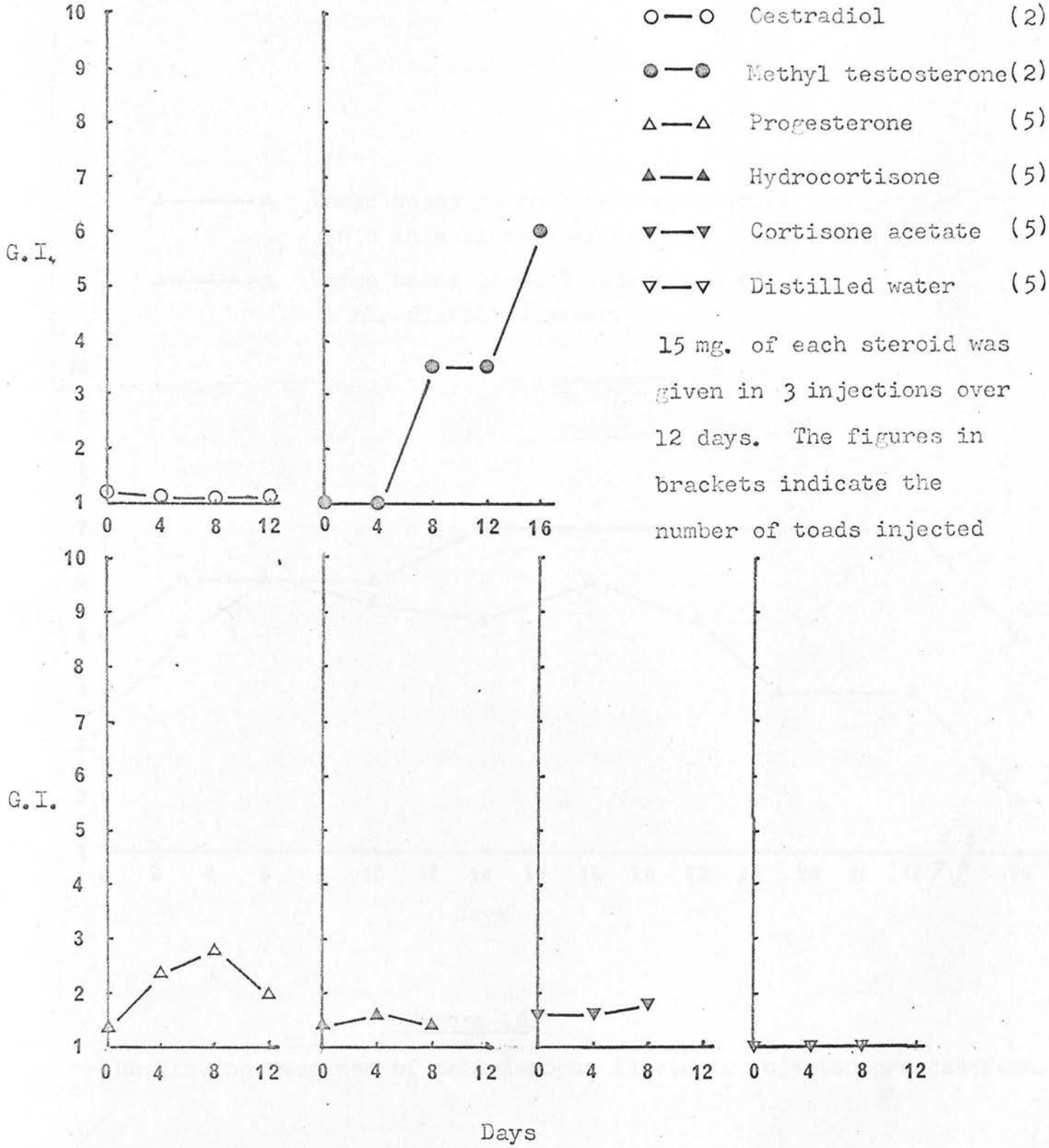

Figure 17.

The gloving response of female Xenopus laevis to injected steroids.

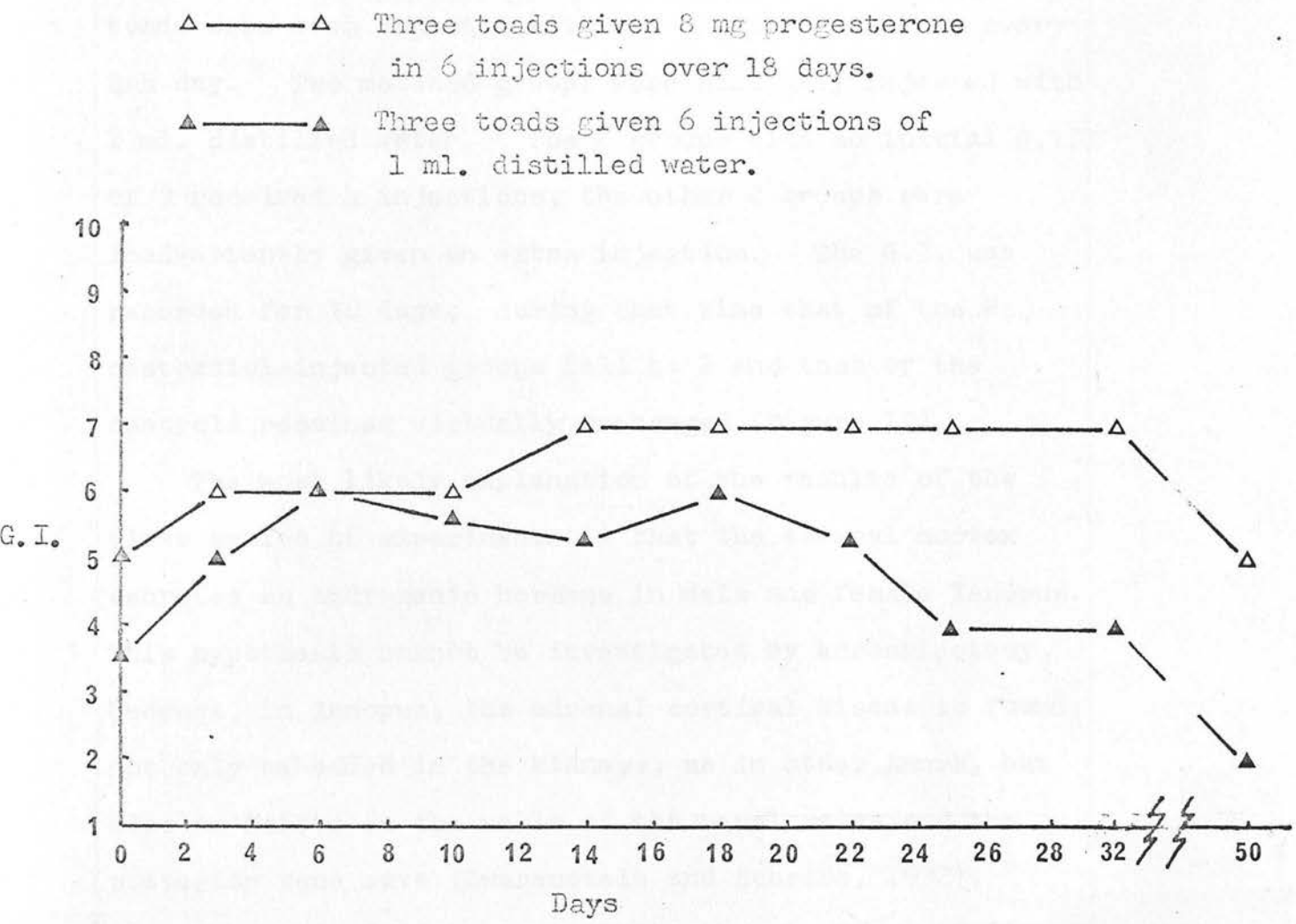

Figure 18.

The gloving response of male Xenopus laevis to injected progesterone.

Oestrogens often antagonise the actions of androgens and in the next experiment the effect of exogenous oestradiol on the gloving of male X. laevis is investigated.

Ten toads with a mean G.I. of 7, and 10 fully gloved toads were each injected with 400 μg. oestradiol on every 4th day. Two matched groups were similarly injected with 1 ml. distilled water. The 2 groups with an initial G.I. of 7 received 4 injections, the other 2 groups were inadvertently given an extra injection. The G.I. was recorded for 30 days; during that time that of the 2 oestradiol-injected groups fell to 2 and that of the controls remained virtually unchanged (Figure 19).

The most likely explanation of the results of the first series of experiments is that the adrenal cortex secretes an androgenic hormone in male and female Xenopus. This hypothesis cannot be investigated by adrenalectomy because, in Xenopus, the adrenal cortical tissue is found, not only embedded in the kidneys, as in other Anura, but also as islets in the walls of the renal veins and the posterior vena cava (Zwarenstein and Schrire, 1932).

It was decided to investigate the gloving activity of the adrenal cortical tissue indirectly. If the androgenic function of the adrenal cortical cells depends on stimulation by pituitary trophic hormones, one would expect adenohypophysectomy to cause complete regression of the gloves of castrated toads.

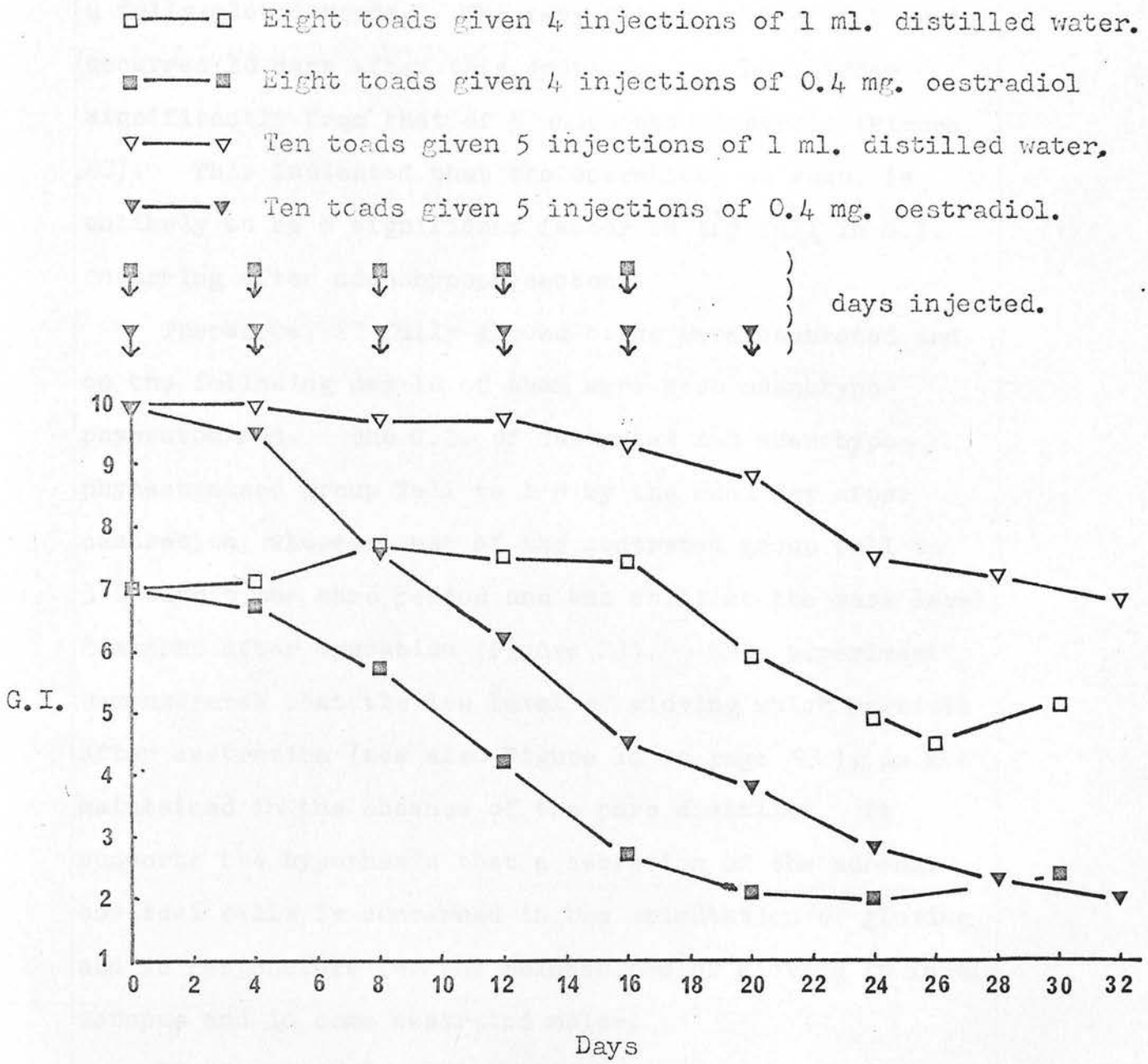

Figure 19.

The gloving response of male Xenopus laevis to injected oestradiol.

The function of the adenohypophysis in gloving.

The effect of the trauma of the operation itself was investigated by performing sham adenohypophysectomy on 4 fully-gloved toads. The very slight fall of G.I. which occurred 10 days after this operation did not differ significantly from that of 5 unoperated controls (Figure 20). This indicated that the operation, as such, is unlikely to be a significant factor in any fall in G.I. occurring after adenohypophysectomy.

Therefore, 20 fully-gloved toads were castrated and on the following day 10 of them were also adenohypophysectomised. The G.I. of castrated and adenohypophysectomised group fell to 1·0 by the 22nd day after castration, whereas that of the castrated group fell to 3·0 within the same period and was still at the same level 2 months after operation (Figure 21). This experiment demonstrates that the low level of gloving which persists after castration (see also Figure 14 on page 93), is not maintained in the absence of the pars distalis. It supports the hypothesis that a secretion of the adrenal cortical cells is concerned in the stimulation of gloving and is responsible for the maintenance of gloving in female Xenopus and in some castrated males.

If the adrenal cortical tissue secretes an androgen in Xenopus, exogenous A.C.T.H. might be expected to stimulate an increased androgen secretion. The next experiment was designed to test this hypothesis.

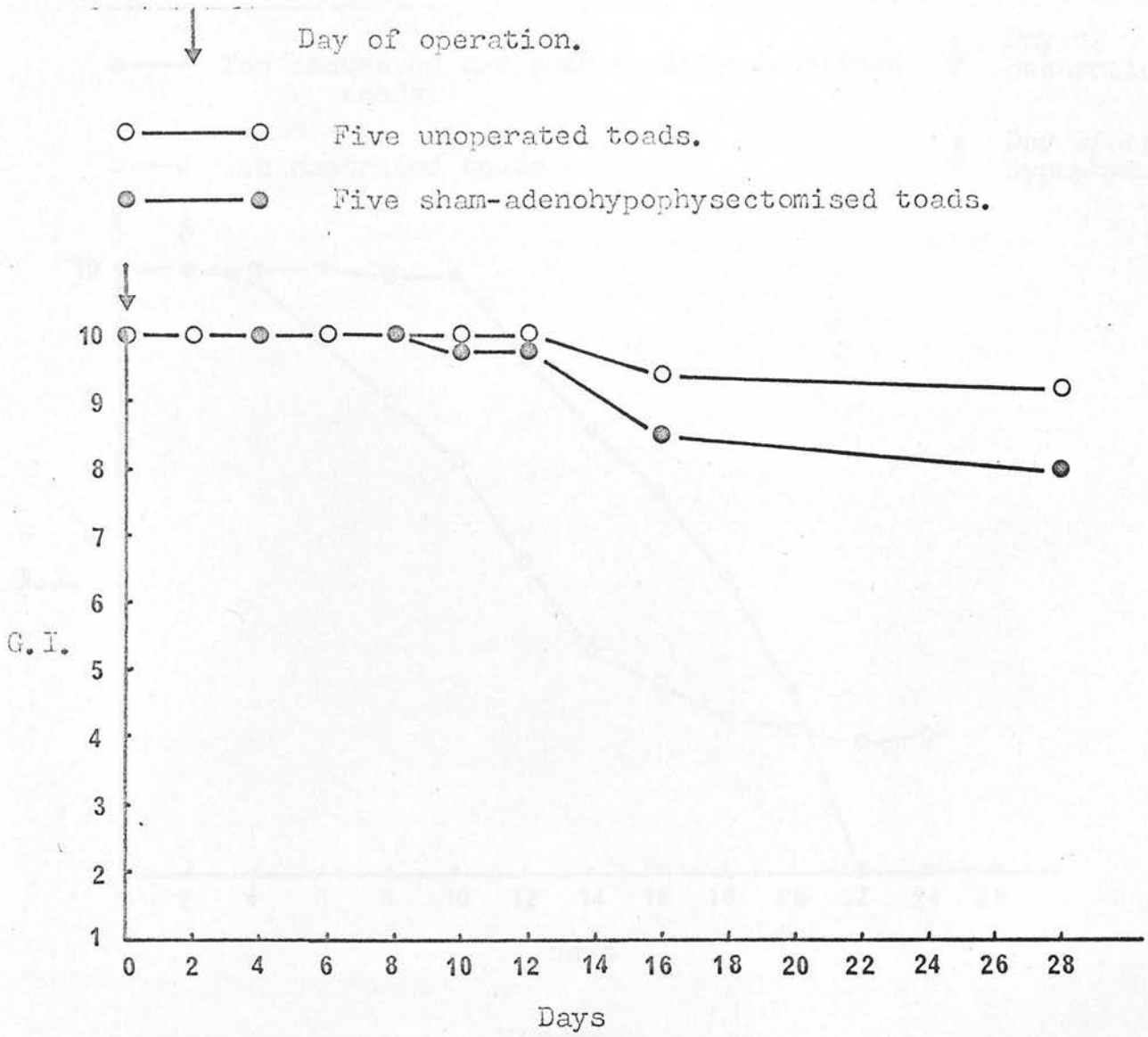

Figure 20.

The gloving response of fully-gloved male Xenopus laevis to sham-adenohypophysectomy.

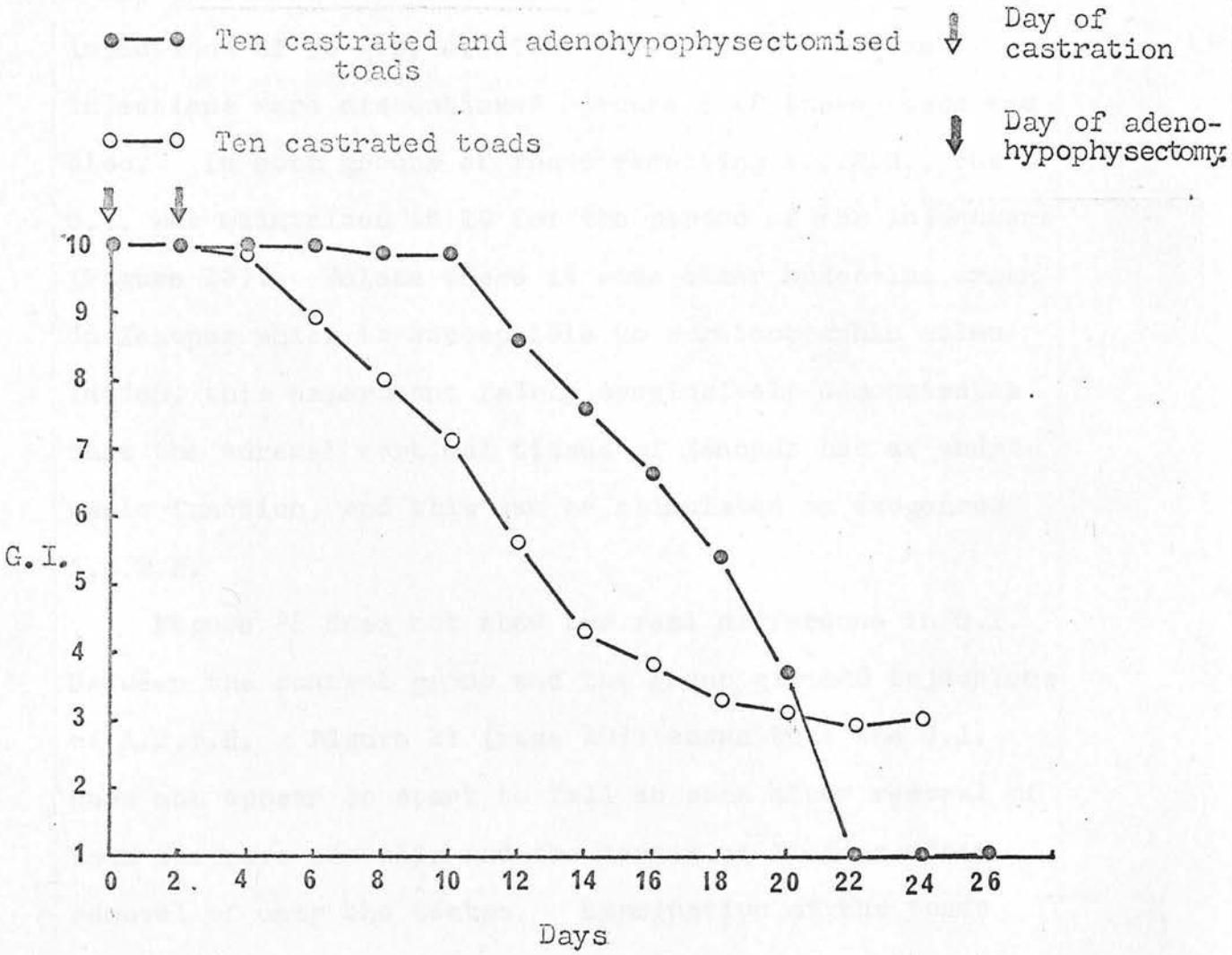

Figure 21.

The gloving response of fully-gloved male Xenopus laevis to castration with and without adenohypophysectomy.

The function of the adrenal cortical tissue in gloving.

Fourteen fully-gloved male toads were castrated and adenohypophysectomised. Five were given 8 daily injections of 30 I.U. A.C.T.H. and 5 received 8 daily injections of 1 ml. distilled water. The other 4 toads received daily injections of 30 I.U. A.C.T.H. for 19 days when the injections were discontinued because 2 of these toads had died. In both groups of toads receiving A.C.T.H., the G.I. was maintained at 10 for the period of the injections (Figure 22). Unless there is some other endocrine organ in Xenopus which is susceptible to corticotrophin stimulation, this experiment fairly conclusively demonstrates that the adrenal cortical tissue of Xenopus has an androgenic function, and this can be stimulated by exogenous A.C.T.H.

Figure 22 does not show the real difference in G.I. between the control group and the group given 8 injections of A.C.T.H. Figure 21 (page 107) shows that the G.I. does not appear to start to fall so soon after removal of both the pars distalis and the testes as it does after removal of only the testes. Examination of the toads showed that this retention of gloving is due to the inhibition of skin shedding which follows adenohypophysectomy (Jørgensen and Larsen, 1964). The apparent rate of fall of the G.I. of the group injected with distilled water was therefore slower than the real rate because of this retention of old skin. In those toads receiving A.C.T.H., however, skin shedding appeared normal and there was no

All toads castrated and adenohypophysectomised on day 0.

●——● Four toads given 19 injections of 30 I.U. A.C.T.H.

■——■ Five toads given 8 injections of 30 I.U. A.C.T.H.

□——□ Five toads given 8 injections of 1 ml. distilled water.

All injections given daily starting on day 0.

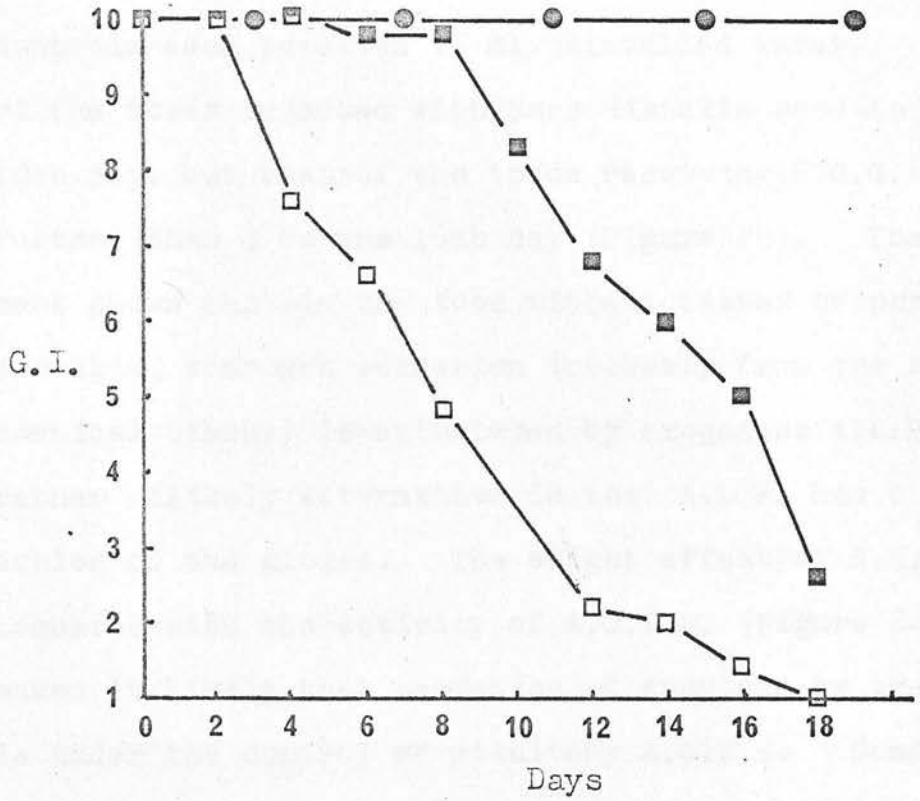

Figure 22.

The gloving response of fully-gloved male Xenopus laevis to injections of A.C.T.H. after castration and adenohypophysectomy.

retention of the original gloves.

In the next experiment the gloving activities of Xenopus A.L.P., H.C.G., testosterone and hydrocortisone on castrated and adenohypophysectomised males are compared. The toads used had been castrated and adenohypophysectomised 3 weeks previously. All the substances were given in 15 daily injections during which time 2 toads each received a total of 15 Xenopus partes distales, 2 received 900 I.U. H.C.G., 2 received 75 mg. methyl testosterone, one received 75 mg. hydrocortisone and 5 controls each received 15 ml. distilled water. The G.I. of the toads injected with pars distalis rose to 8 by the 10th day, but that of the toads receiving H.C.G. rose no further than 3 on the 16th day (Figure 23). The experiment shows that in the toad without testes or pars distalis, androgen secretion (probably from the adrenal cortical tissue) is stimulated by exogenous A.L.P. The rather unlikely alternative is that A.L.P. has a direct action on the gloves. The slight effect of H.C.G., compared with the activity of A.C.T.H. (Figure 22, page 109) makes it likely that secretion of androgen by the adrenals is under the control of pituitary A.C.T.H. Compared with its effect in the castrated toad, (Figures 15 & 16, and Table 9, pages 96, 97 & 99) testosterone had little effect but more activity was shown by hydrocortisone. A possible explanation is that the activity of the adrenals is maintained by the pars distalis in castrated toads, but not in

toads also adenohypophysectomised; injected steroids may act as precursors in the synthesis of the natural androgen and have a greater effect in toads with an already active adrenal cortical tissue. From their relative activities in this experiment, it seems likely that hydrocortisone is a better intermediate in this synthesis than is testosterone.

In the next experiment the effect of hydrocortisone was tested on recently castrated and adenohypophysectomised fully-gloved males. Nine fully-gloved toads were castrated and adenohypophysectomised. Three received a single injection of 6 mg. hydrocortisone sodium succinate, 3 received 13 daily injections of the same substance and 3 received an equal number of injections of 1 ml. distilled water. The G.I. of the 2 groups injected with hydrocortisone fell slightly more slowly than that of the distilled water controls; the greatest difference was between the controls and those given a single injection of 6 mg. hydrocortisone (Figure 24). Even when the trophic effect of the adenohypophysis has only just been removed, hydrocortisone has little effect in maintaining gloving in castrated toads. This agrees with the idea that it may act as a precursor in the synthesis of an adrenocortical androgen.

The comparative effects of gonadotrophins on gloving.

The next experiments were designed to investigate whether the secretion of testicular androgen is stimulated

All toads castrated and adenohypophysectomised 3 weeks before day 0.

●—● Two toads each given a total of 15 Xenopus adenohypophyses.

○—○ One toad given a total of 75 mg. hydrocortisone.

▼—▼ Two toads each given a total of 900 I.U. H.C.G.

□—□ Two toads each given a total of 75 mg. methyl testosterone.

■—■ Five toads each given a total of 15 ml. distilled water.

↓ Days injected

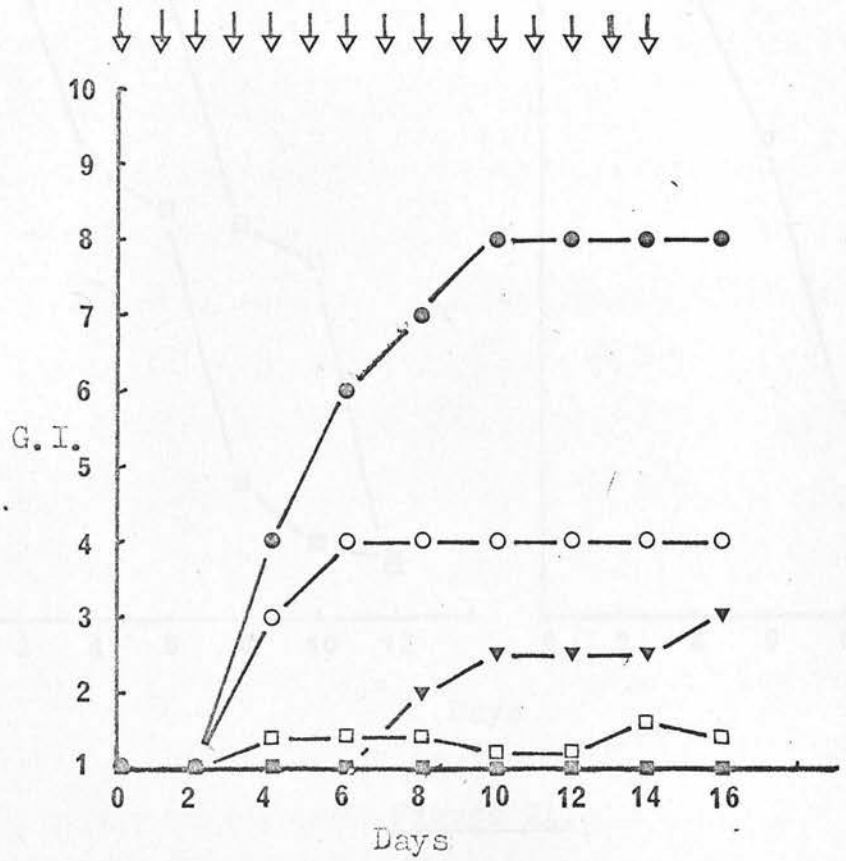

Figure 23.

The gloving response of ungloved castrated and adenohypophysectomised male Xenopus laevis to injections of 2 gonadotrophins, an androgen and an adrenocorticosteroid.

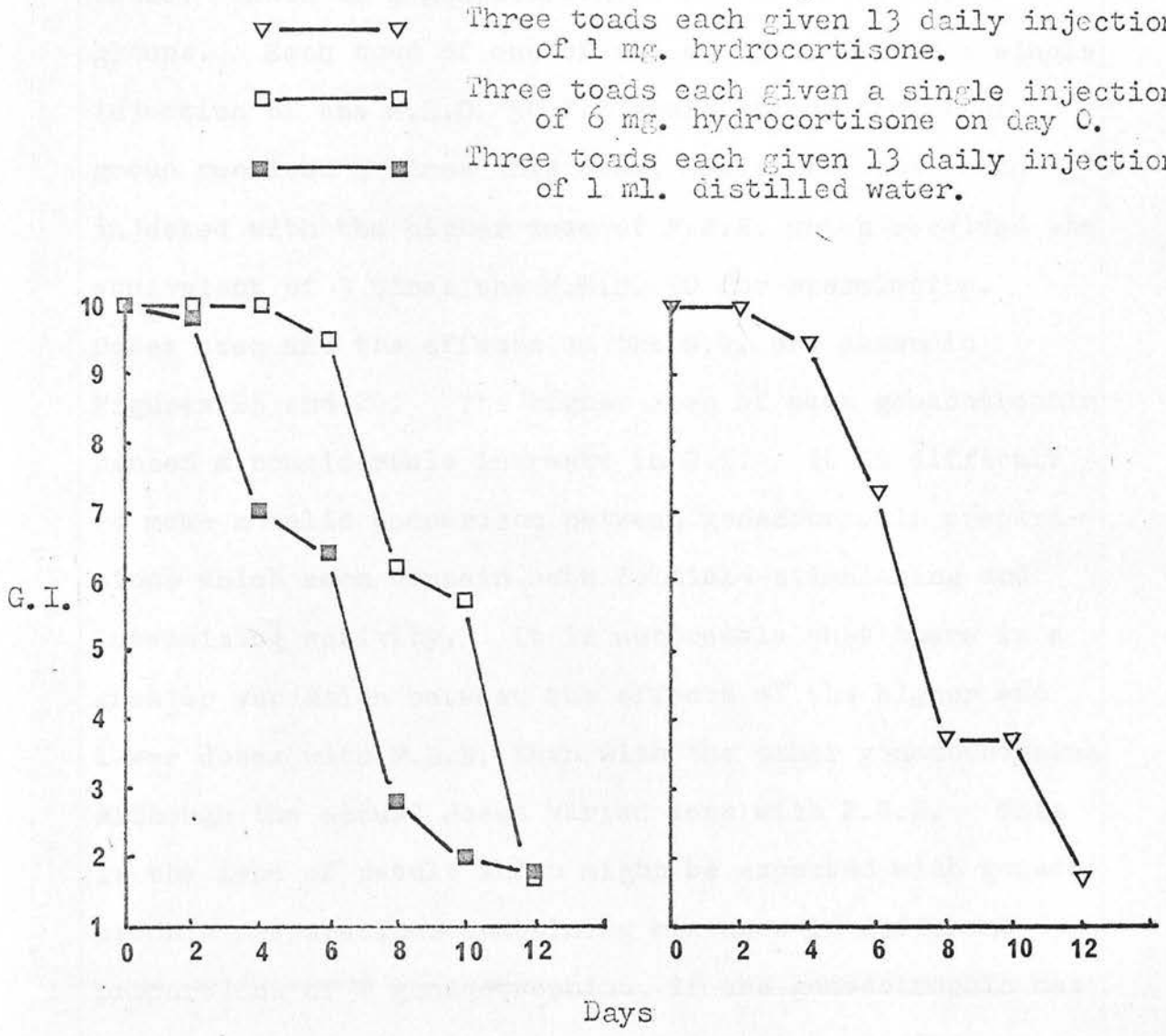

Figure 24.

The gloving response of fully-gloved male Xenopus laevis to castration and adenohypophysectomy and injections of hydrocortisone.

preferentially by a follicle-stimulating or a luteinizing type of gonadotrophin. Forty intact males with little gloving were selected and put into matched groups of 5 toads. Each of 4 gonadotrophins was injected into 2 groups. Each toad of one of the groups received a single injection of the M.E.D. 50 for spermiation, the other group received 4 times this dose, except for the group injected with the higher dose of F.S.H. which received the equivalent of 3 times the M.E.D. 50 for spermiation. Doses used and the effects on the G.I. are shown in Figures 25 and 26. The higher does of each gonadotrophin caused a considerable increase in G.I. It is difficult to make a valid comparison between gonadotrophin preparations which each contain both follicle-stimulating and luteinizing activity. It is noticeable that there is a greater variation between the effects of the higher and lower doses with F.S.H. than with the other gonadotrophins, although the actual doses varied less with F.S.H. This is the type of result which might be expected with gonadotrophic preparations containing mixtures in different proportions of 2 gonadotrophins, if one gonadotrophin has greater spermiation-inducing activity than gloving activity and vice-versa.

To eliminate any effect of endogenous gonadotrophin, the gloving activities of H.C.G. and F.S.H. were compared on adenohypophysectomised toads in the next experiment.

■——■ Five toads injected with 4.5 mg. ox A.L.P.

□——□ Five toads injected with 1.1 mg. ox A.I.P.

▼——▼ Five toads injected with 60 I.U. H.C.G.

▽——▽ Five toads injected with 15 I.U. H.C.G.

All injections given on day 0.

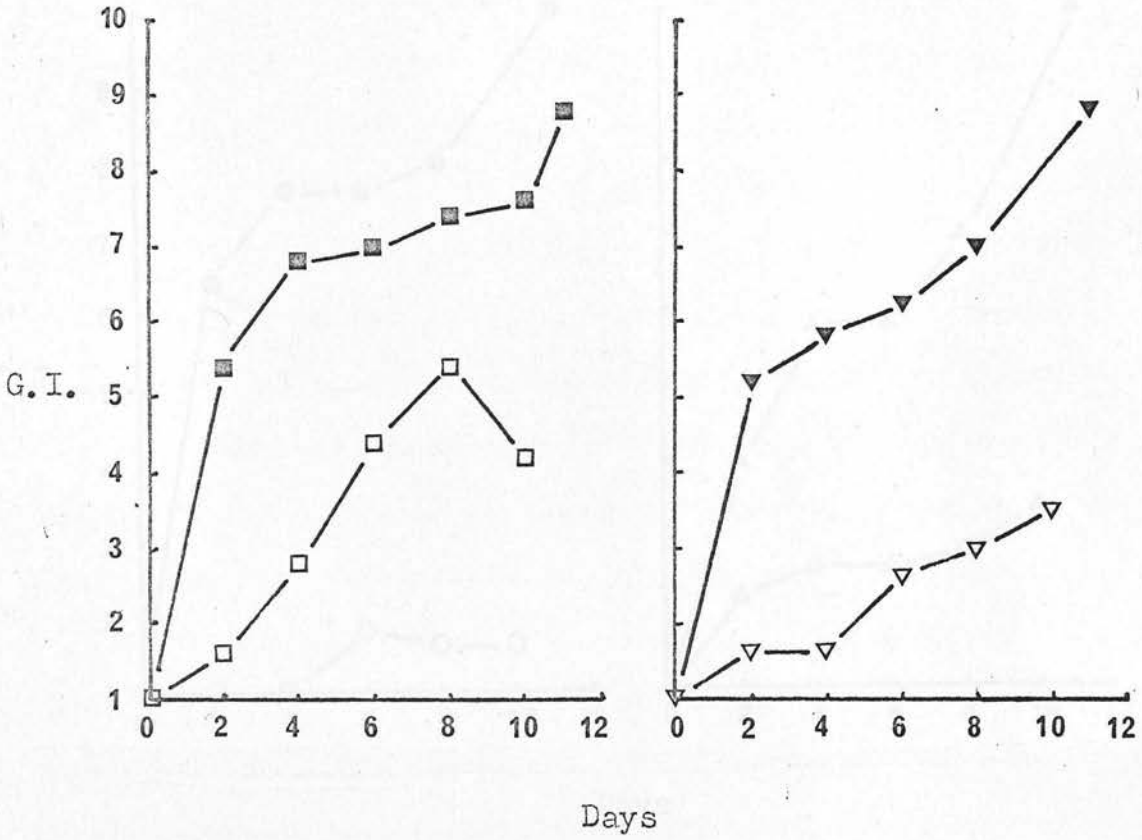

Figure 25.

The gloving response of ungloved male Xenopus laevis to injections of ox A.L.P. and H.C.G.

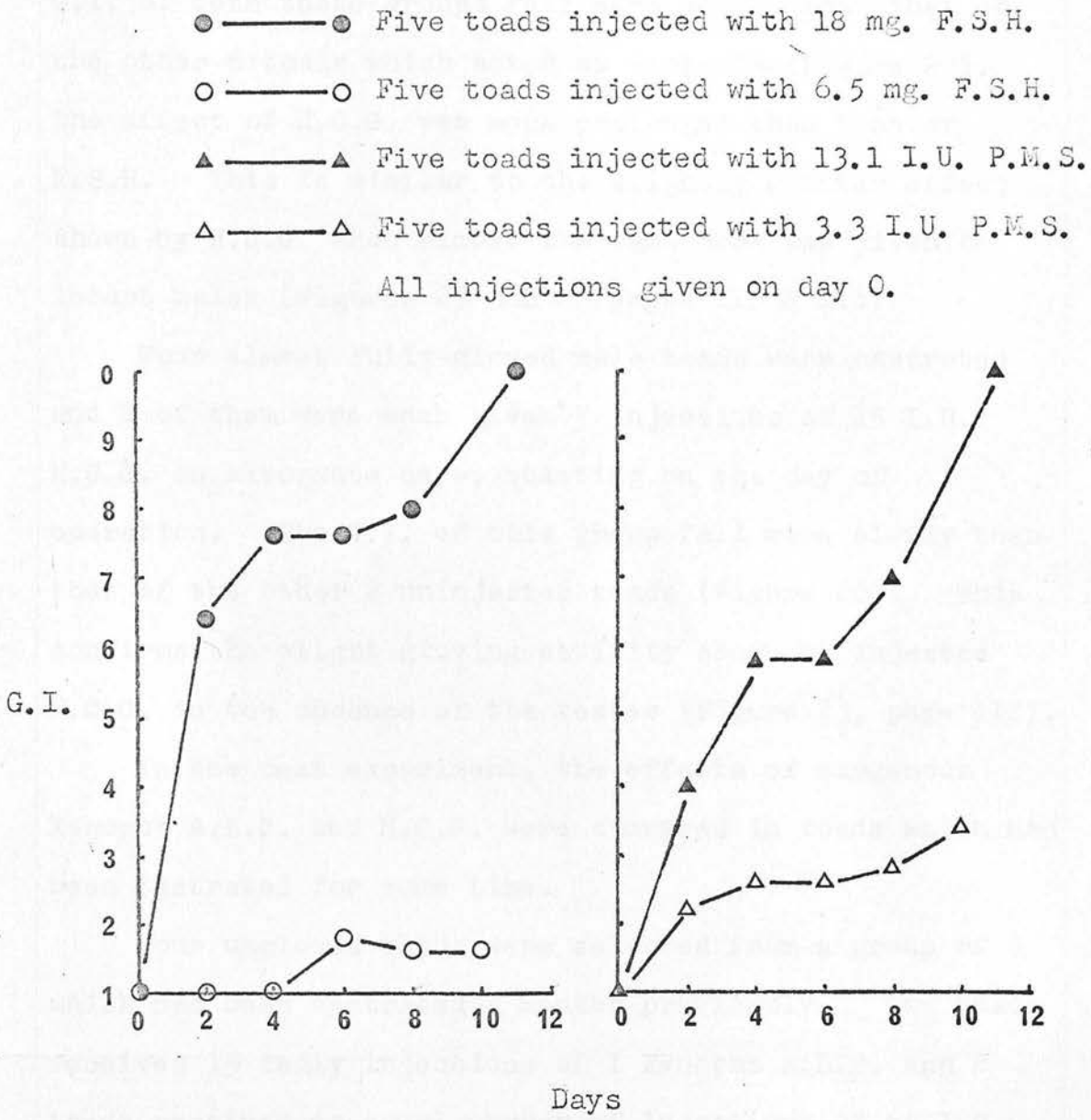

Figure 26.

The gloving response of ungloved male Xenopus laevis to injections of F.S.H. and P.M.S.

Sixteen fully-gloved males were adenohypophysectomised and 2 groups of 5 toads were each given a single injection of 7 mg. F.S.H. and 15 I.U. H.C.G. respectively. The G.I. of both these groups fell more slowly than that of the other 6 toads which acted as controls (Figure 27). The effect of H.C.G. was more prolonged than that of F.S.H. This is similar to the slightly greater effect shown by H.C.G. when almost the same dose was given to intact males (Figures 25 and 26, pages 115 & 116).

Four almost fully-gloved male toads were castrated and 2 of them were each given 7 injections of 15 I.U. H.C.G. on alternate days, starting on the day of operation. The G.I. of this group fell more slowly than that of the other 2 uninjected toads (Figure 28). This confirms the slight gloving activity shown by injected H.C.G. in the absence of the testes (Figure 23, page 112).

In the next experiment, the effects of exogenous Xenopus A.L.P. and H.C.G. were compared in toads which had been castrated for some time.

Four ungloved toads were selected from a group of 9 which had been castrated 2 months previously. Two toads received 15 daily injections of 1 Xenopus A.L.P. and 2 toads received an equal number of injections of 60 I.U. H.C.G. The other 5 castrated toads, which had a G.I. of 3·8, were used as uninjected controls. The G.I. of the group receiving H.C.G. rose more than that of the group injected with Xenopus A.L.P. The G.I. of the controls

All toads adenohypophysectomised on day 0.

□——□ Five toads injected with 15 I.U. H.C.G. on day 0.

□——□ Five toads injected with 7 mg. F.S.H. on day 0.

□——□ Six uninjected toads.

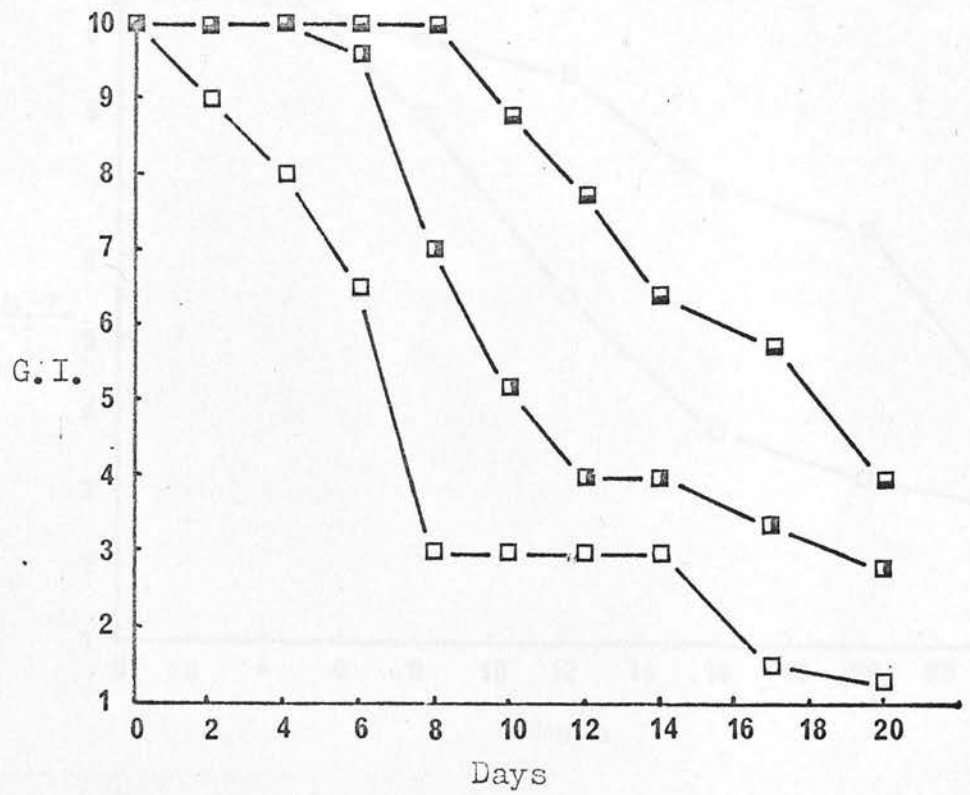

Figure 27.

The gloving response of fully-gloved male Xenopus laevis to adenohypophysectomy and injection of H.C.G. and F.S.H.

All toads castrated on day 0.

■——■ Two toads given 7 injections of 15 I.U. H.C.G.

□——□ Two uninjected toads.

All injections given on alternate days starting on day 0.

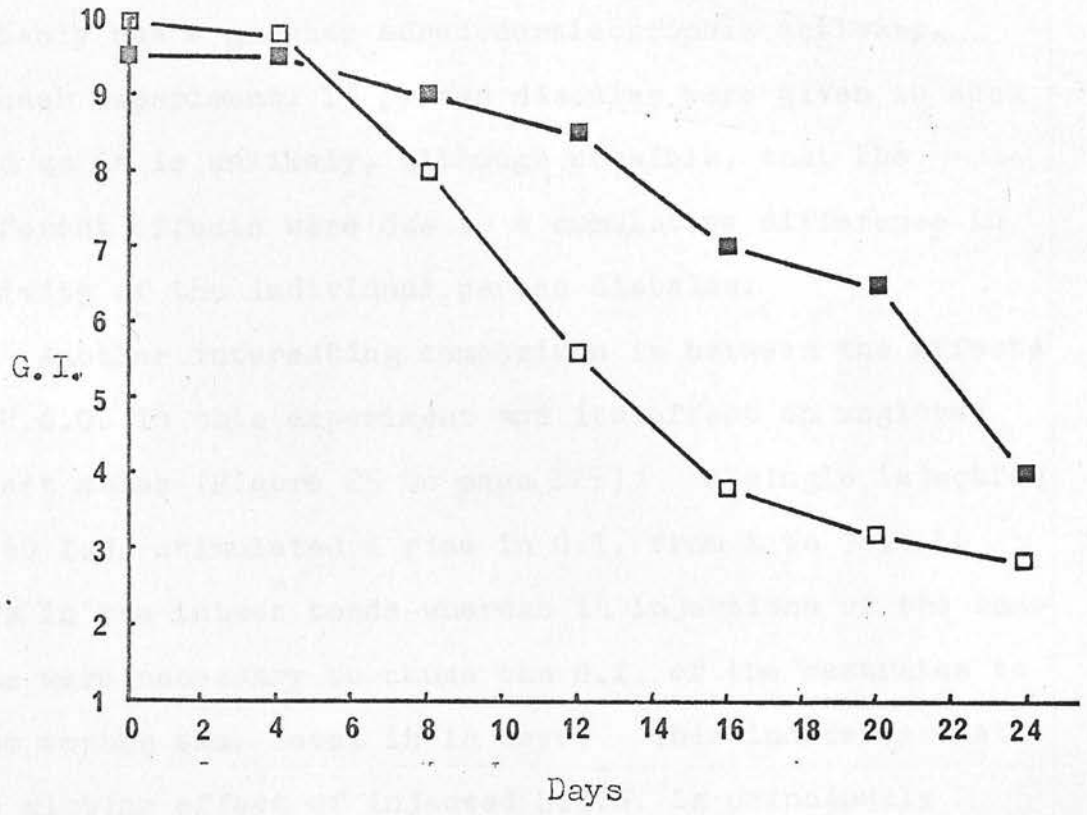

Figure 28.

The gloving response of fully-gloved male Xenopus laevis to castration and injections of H.C.G.

altered by only 0·2 during the experiment and is not plotted on the graph for the sake of clarity (Figure 29).

It is interesting to compare these effects of Xenopus A.L.P. and H.C.G. on castrated males, with the effects of equal doses on castrated and adenohypophysectomised toads (Figure 23 on page 112). In the doses used, it appears that H.C.G. has a greater gonadotrophic activity on the testes, but that, in the absence of the testes, Xenopus A.L.P. has a greater effect on gloving. Xenopus A.L.P. probably has a greater adrenocorticotrophic activity. In each experiment, 15 partes distales were given to each toad so it is unlikely, although possible, that the different effects were due to a cumulative difference in activity of the individual partes distales.

Another interesting comparison is between the effects of H.C.G. in this experiment and its effect on ungloved intact males (Figure 25 on page 115). A single injection of 60 I.U. stimulated a rise in G.I. from 1 to 9 in 10 days in the intact toads whereas 14 injections of the same dose were necessary to cause the G.I. of the castrates to rise to the same level in 14 days. This indicates that the gloving effect of injected H.C.G. is principally mediated through the testes.

In another experiment, the effect of H.C.G. on castrated males is compared with its effect on castrated and adenohypophysectomised males.

All toads castrated 2 months before day 0.

●——● Two toads given 15 daily injections of 1 Xenopus adenohypophysis.

○——○ Two toads given 15 daily injections of 60 I.U. H.C.G.

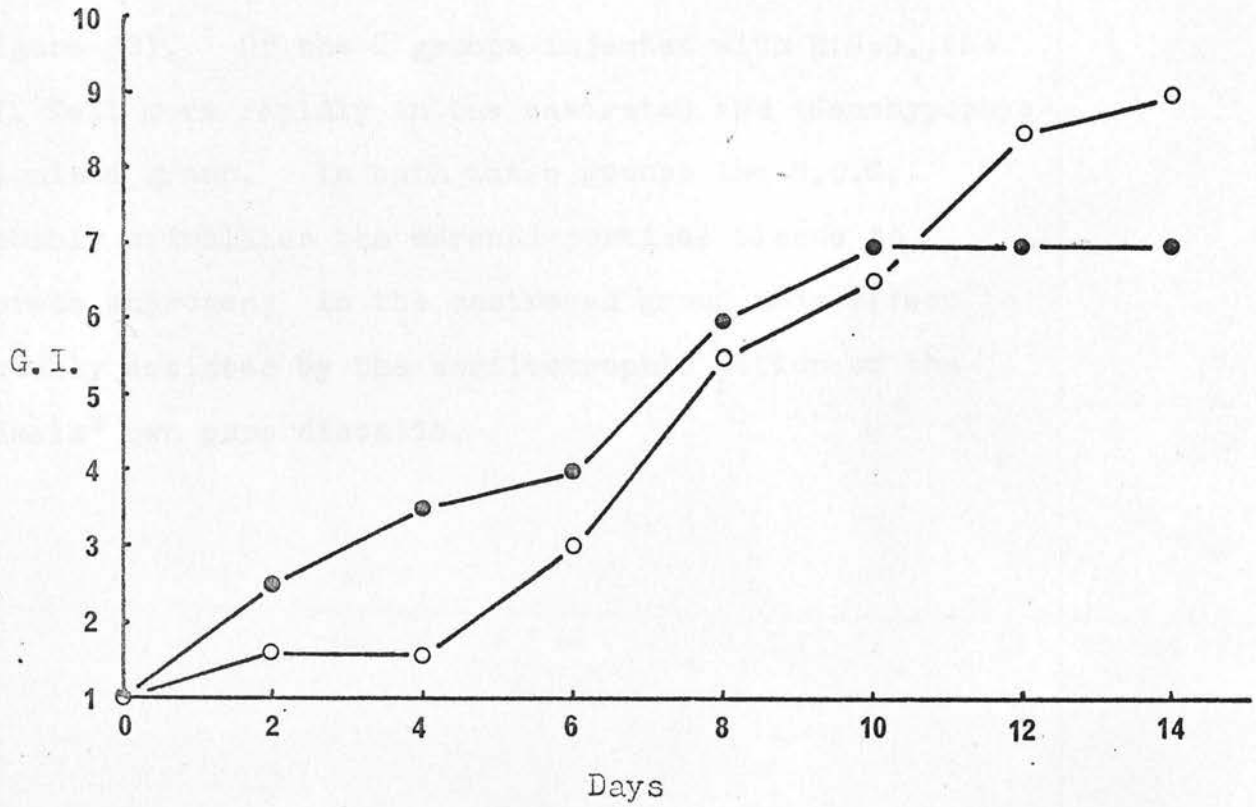

Figure 29.

The gloving response of castrated male Xenopus laevis to injections of H.C.G. and Xenopus adenohypophyses.

Six almost fully-gloved toads were castrated and 4 of them were also adenohypophysectomised. The 2 castrated toads and 2 of the castrated and adenohypophysectomised toads were each given 7 injections of 15 I.U. H.C.G. on alternate days. The other 2 castrated and adenohypophysectomised toads acted as controls and received 15 injections of 1 ml. distilled water. The G.I. of both the groups injected with H.C.G. fell more slowly than that of the group receiving distilled water (Figure 30). Of the 2 groups injected with H.C.G., the G.I. fell more rapidly in the castrated and adenohypophysectomised group. In both these groups the H.C.G. probably stimulates the adrenal cortical tissue to secrete androgen; in the castrated group this effect is probably assisted by the corticotrophic action of the animals' own pars distalis.

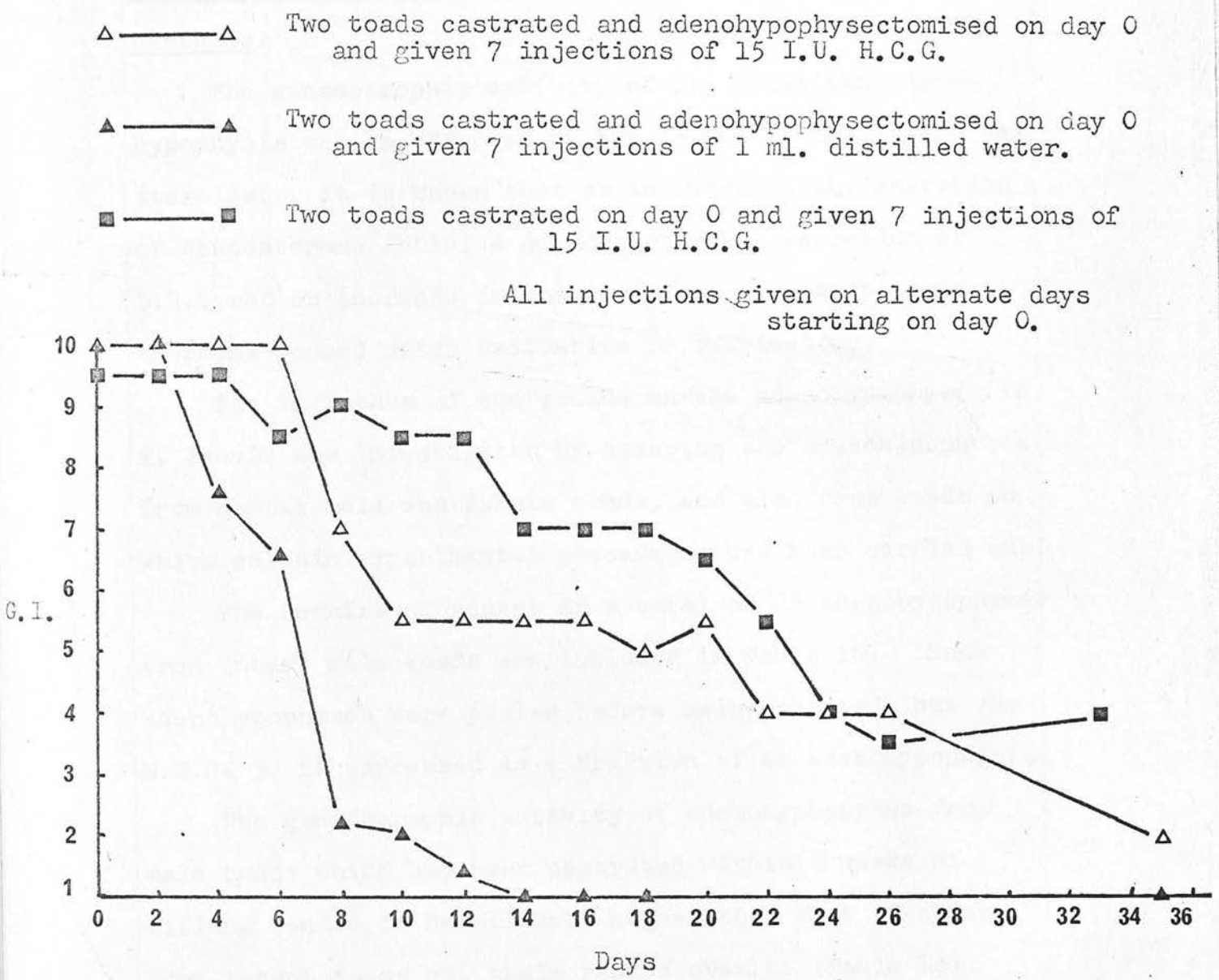

Figure 30.

The gloving response of fully-gloved male Xenopus laevis to castration with and without adenohypophysectomy followed by injections of H.C.G.

THE MALE AND FEMALE ADENOHYPOPHYSIS.

Factors affecting the gonadotrophin content of the pars distalis.

The gonadotrophic activity of the Mammalian adenohypophysis can be affected by the level of circulating sex steroids. It is known that an increase in the secretion of testosterone inhibits adenohypophyseal secretion of L.H., and an increase in the level of circulating gonadotrophins occurs after castration or ovariectomy.

The influence of the gonads on the adenohypophysis in X. laevis was investigated by assaying the adenohypophyses from normal male and female toads, and also from toads on which certain experimental procedures had been carried out.

The results of assays of a total of 29 adenohypophyses from intact male toads are included in Table 10. Some adenohypophyses were pooled before being assayed, but the M.E.D. 50 is expressed as a fraction of an adenohypophysis.

The gonadotrophic activity of adenohypophyses from male toads which had been castrated within 8 weeks of killing tended to be slightly higher than that of those from intact toads but their ranges overlap (Table 10).

The gonadotrophic activity of adenohypophyses from toads castrated for over 2 years was at least 7 times greater than that of intact males. This demonstrates that in the normal toad there is a negative feed-back system by means of which endocrine secretions of the

TABLE 10.

Gonadotrophin assays of adenohypophyses from male Xenopus laevis.

Toads						Biological assay				
Condition				Number	Mean body wt. (g.)	Mean A.L.P. wt. (mg.)	M.E.D. 50 spermiation (S) Fraction of one A.L.P.	M.E.D. 50 spermiation (S) Weight of A.L.P. (µg.)	M.E.D. 50 ovulation (O) Fraction of one A.L.P.	M.E.D. 50 ovulation (O) Weight of A.L.P. (µg.)	$\frac{1}{S} : \frac{1}{O}$
Intact	uninjected		G.I.4	5	46·0	0·560	0·04	22·5	-	-	-
			G.I.4	5	41·0	0·714	0·05	173·8	-	-	-
			G.I.5·5	6	49·0	0·761	0·041	30·94	-	≏1,305	≏42:1
			G.I.7	2	48·0	0·946	0·015	13·9	-	> 63·1 <732·0	≏ or >37:1
			G.I.10	5	62·0	0·739	0·021	78·63	-	-	-
	injected with:-	aq.dest 15 x 1 ml.	G.I.8·5	2	44·0	0·826	0·014	11·77	-	>165·0 <330·0	≏ 17:1
		Xenopus saline 3x1 ml.	G.I.1·0	2	47·5	0·956	0·028	26·45	-	-	-
		Xenopus saline 10x1 ml.	G.I.2·5	2	42·5	0·562	0·031	17·28	-	>225·0	>11.1
		testosterone 7 x 1 mg.		1	35·5	0·980	0·065	63·92	-	-	-
		testosterone 16 x 1 mg.		2	48·0	0·622	0·027	16·82	-	>249·0	>14:1
		hydrocortisone 8 x 1 mg.		2	47·0	2·032	>0·40	>813·0	-	-	-
		hydrocortisone 15 x 1 mg.		2	43·0	0·443	0·058	28·72	-	-	-
Castrated	1 week			2	59·0	1·086	0·014	14·78	-	-	-
	2 weeks			2	64·0	0·918	0·020	18·53	-	-	-
	4 weeks			1	51·0	1·369	0·0089	12·16	-	>136·9	>11:1
	8 weeks			1	32·0	0·456	0·013	6·03	-	-	-
	2 years			1	92·0	2·255	0·00078	1·77	-	-	-
	6 years			1	66·0	1·490	0·00083	1·244	0·071	105·3	108·6:1
	6 years			1	75·0	1·271	0·0020	2·542	0·021	263·9	103·6:1
	6 years			1	81·0	1·290	0·0011	1·460	0·061	78·21	43:1

testes inhibit pituitary gonadotrophin secretion; this inhibition is removed by castration.

The activity of the adenohypophyses from males which had received 16 injections of 1 mg. testosterone was within the range of activities of adenohypophyses from normal males which had not been injected or had been injected with distilled water or with Xenopus saline. However, one adenohypophysis from a male which received 7 injections of 1 mg. testosterone was less active and just outside the range for normal adenohypophyses (Table 10).

The adenohypophyses from 2 toads which were each given 8 injections of 1 mg. hydrocortisone were so much less active than normal adenohypophyses that an M.E.D. 50 could not be obtained (Table 10). This may indicate that hydrocortisone is more like the natural Xenopus testicular androgen than is testosterone. The greater gloving activity of exogenous hydrocortisone compared with testosterone in castrated and hypophysectomised males would seem to confirm this (Figure 23 on page 112).

The inhibitory effect of both hydrocortisone and testosterone appears to get less when injections continue for a longer period. Adenohypophyses from toads which had received a longer course of injection of testosterone and of hydrocortisone had a greater gonadotrophic activity than those from toads which had fewer injections (and a smaller dose) of each steroid (Table 10).

It is interesting that the ratio of spermiation/

ovulation activities of 2 adenohypophyses from castrated males was over double the 2 ratios obtained for adenohypophyses from intact males. Ovulation in X. laevis is not stimulated by a follicle-stimulating type of gonadotrophin, whereas spermiation is stimulated by F.S.H. and L.H. Therefore, it appears that the adenohypophyses from castrated males tend to have a greater increase in F.S.H. content than in L.H. content, compared with adenohypophyses from intact toads.

The results of assays of adenohypophyses from female X. laevis are listed in Table 11. In general, female adenohypophyses were more active than those of males, either on a weight for weight basis or when total activities of adenohypophyses are compared (Table 11). There was a similar increase in activity after gonadectomy, but the activities of adenohypophyses from toads injected with oestradiol was within the range of those from normal females. No difference was found in the activity of an adenohypophysis removed after ovulation following injection of H.C.G.

The spermiation/ovulation ratios obtained showed the same tendency as those for male adenohypophyses. A low ratio was found in adenohypophyses from females injected with oestrogen and a high ratio in an adenohypophysis from an ovariectomised toad. It appears that the effect of the testes in the male, and the ovaries in the female, is more potent in inhibiting F.S.H. than in inhibiting L.H. secretion.

TABLE 11.

Gonadotrophin assays of adenohypophyses of female Xenopus laevis.

Toads						Biological Assay				
Condition			Number	Mean body weight (g)	Mean A.L.P. wt. (mg.)	M.E.D.50 spermiation (S)		M.E.D.50 ovulation (O)		$\frac{1}{S} : \frac{1}{O}$
						Fraction of one A.L.P.	Weight of A.L.P. (µg.)	Fraction of one A.L.P.	Weight of A.L.P. (µg.)	
Intact	uninjected	mature ovaries	2	45·5	0·755	0·0049	3·68	-	-	-
		atrophic ovaries	3	50·0	0·858	0·0072	6·173	0·42	359·0	58:1
		sick toads with bilateral cataract	1	73·0	1·010	0·015	15·5	-	-	-
			1	87·0	1·154	0·013	15·19	-	-	-
	injected with oestradiol	10xlmg.	2	93·0	1·407	0·019	26·19	-	> 281·4 < 562·8	14:1
		20xlmg.	1	118·0	0·768	0·0040	6·21	-	> 153·6	>24:1
		70 I.U. H.C.G.	1	74·0	0·855	0·0085	7·288	-	> 171·0	>23:1
Ovariectomised 6 years		fragment of ovary left	1	85·0	3·33	0·00021	0·7091	0·18	58·86	97·6:1
			1	115·0	1·77	0·0013	2·309	-	-	-

THE FEMALE.

In-vitro ovulation.

It is well established that X. laevis ovulate and oviposit when injected with L.H. Certain steroids have also been shown to cause egg laying when injected into Xenopus, and to cause ovulation when administered to its excised ovary (Shapiro and Zwarenstein, 1937; Zwarenstein, 1937; Shapiro, 1939; Burgers and Zwarenstein, 1955). Gonadotrophic preparations have been found to cause ovulation of the excised ovaries of other species of Anura, but their in-vitro activity on Xenopus ovaries has not been tried. The following experiments were done to see if substances which cause ovulation, when injected into X.laevis, will also do so when administered to the excised ovary.

In pilot experiments, ovaries were removed and each divided into not more than 6 pieces. Each piece was put into a Petri dish containing 20 ml. Xenopus saline, a fluid which is isotonic with Xenopus tissues (Landgrebe and Waring, 1950), to which the substances being tested were added. The ovarian pieces were watched for 48 hours and it was found that, when ovulation occurred, it did so within 24 hours. In subsequent experiments, ovulation within 24 hours became the criterion of a positive response. To ensure that ovaries were capable of ovulating, all those used for in-vitro experiments were taken from toads which had ovulated in response to an injection of 70 I.U.

H.C.G. In the doses tried, Xenopus A.L.P. did not by itself cause ovulation, but did so when given with cortisone. Cortisone and H.C.G. caused ovulation both when given alone and together (Table 12).

In subsequent in-vitro ovulation experiments, a different apparatus was used (Plate VIII). Each ovary was suspended in a separate tissue bath containing 40 ml. Xenopus saline. This was aerated by a gentle stream of oxygen bubbles. The outlet at the bottom was off-set to allow ova to collect where they could be easily seen. Twelve ovary baths were maintained at $22^{\circ}C \pm 0{\cdot}5^{\circ}C$ in a thermostatically controlled water-bath. The ovary baths and associated glassware and tubing was sterilized by autoclaving before each experiment. Eight thousand I.U. penicillin G was added to the saline in each tube to prevent the growth of any bacteria introduced with the ovaries. In this solution, ovaries remained in good condition for several days, during which time ovaries in saline without penicillin disintegrated into a thick soup.

Before investigating the effects of various steroid and protein hormones, the effect of altering the concentration of the Xenopus saline was tested. It was found that hypertonic saline up to 1·5 times the normal concentration did not cause ovulation. Hypotonic Xenopus saline containing half the normal concentration of salts caused shedding of ova. This result showed that, in the

TABLE 12.

In-vitro ovulation of fragments of Xenopus laevis ovaries.

Dose of material added to 20 ml. Xenopus Saline			Number of ovaries	
A.L.P. (fraction)	H.C.G. (I.U.)	Cortisone ac. (mg.)	ovulated	used
½	-	-	0	4
1	-	-	0	1
½	-	5	1	1
1	-	10	2	2
-	70	-	1	3
-	140	-	1	3
-	280	-	2	2
-	70	5	1	2
-	140	5	0	1
-	140	10	2	2
-	280	10	2	2
-	-	5	0	1
-	-	10	1	1
-	-	-	0	4

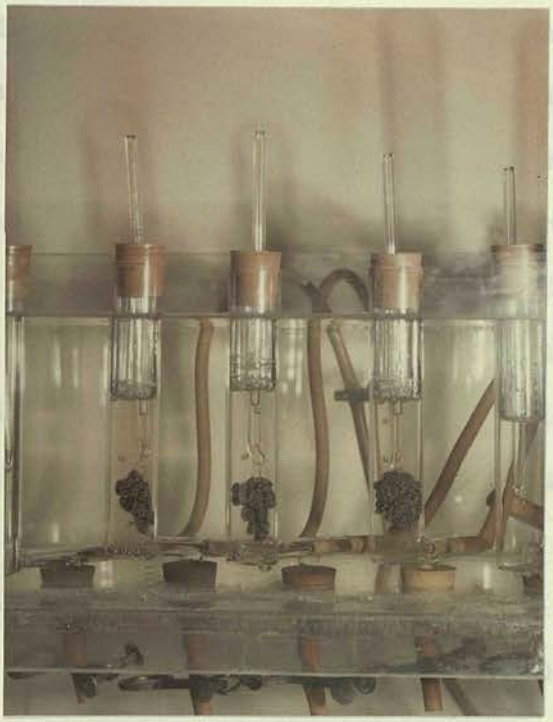

PLATE VIII.

Apparatus used for in-vitro ovulation.

concentrations to be used, no hormone would cause ovulation through its osmotic effect.

The effect of gonadotrophins on the excised ovary.

Excised ovaries were immersed in Xenopus saline containing Xenopus A.L.P. and H.C.G. in various concentrations (Tables 13 & 14). It will be seen that both gonadotrophic preparations caused ovulation, and the lowest effective doses were $\frac{1}{2}$ Xenopus A.L.P. and 175 I.U. H.C.G. These results indicate that these gonadotrophins directly stimulate ovulation, but the amount needed in 40 ml. saline to stimulate ovulation is greater than that which causes ovulation when injected. The M.E.D. 50 for in-vivo ovulation in X. laevis weighing between 50 and 77 g. is 29 I.U. H.C.G. (Hobson, 1952). Examination of Table 14 shows that the in-vitro figure is about 20 times greater.

It has been suggested that hyaluronidase is involved in the liberation of sperm in male Anura (van Oordt, 1962). It is possible that it has a similar function in the expulsion of the ovum from the ovarian follicle. The next experiments investigated its activity. Two dose levels were tried, alone and in combination with various doses of H.C.G. (Table 15). In the doses used, hyaluronidase caused no ovulation and had no significant effect on the response to H.C.G. (Table 15).

The possibility that, when ovulation occurs in-vivo,

TABLE 13.

In-vitro ovulation of Xenopus laevis ovaries in response to Xenopus A.L.P.

A.L.P. (fraction)	Number of ovaries	
	ovulated	used
½	1	2
1/4	0	2
1/8	0	2
1/16	0	2
1/32	0	2
nil	0	2

TABLE 14.

In-vitro ovulation of Xenopus laevis ovaries in response to H.C.G.

H.C.G. (I.U.)	Number of ovaries	
	ovulated	used
1,400	11	13
700	5	9
350	2	7
175	1	5
87·5	0	2
nil	0	6

TABLE 15.

The effect of hyaluronidase on the in-vitro ovulation response of Xenopus laevis ovaries to H.C.G.

Added to ovary bath		Number of ovaries	
hyaluronidase (I.U.)	H.C.G. (I.U.)	ovulated	used
nil	nil	0	1
1,500	nil	0	1
3,000	nil	0	1
1,500	175	0	1
1,500	350	0	2
1,500	700	1	1
nil	175	0	1
nil	350	0	2
nil	700	0	1
nil	1,400	2	2

hyaluronidase is liberated earlier in the process, was investigated by injecting hyaluronidase 6 hours before removal of the ovaries. In the doses used, hyaluronidase had no significant effect on the in-vitro ovulation response to H.C.G. and caused no ovulation of ovaries put into Xenopus saline (Table 16).

Female X. laevis ovulate in response to injected gonadotrophin only when it contains luteinizing activity (Hobson, 1965). However, injection of P.M.S. caused an increased response to a "priming" dose of H.C.G. in groups of toads which had previously consistently failed to ovulate when "primed". (Hobson, personal communication). This indicates that the ovulation in response to injected H.C.G. might normally be assisted by endogenous F.S.H. The effect of P.M.S. on the in-vitro response to H.C.G. was next tried. Toads were injected with various doses of P.M.S. 18 hours before their ovaries were removed and set up in Xenopus saline containing various doses of H.C.G. (Table 17). With an interval of 18 hours, the doses of P.M.S. tried had no effect on the response of the excised ovaries to H.C.G. (Table 17). This is perhaps not surprising as in-vitro ovulation is not caused by doses of up to 700 I.U. P.M.S.

It was considered possible that contaminants in the preparation of H.C.G. used might inhibit ovulation in the excised ovary. This was investigated by comparing the in-vitro activities of laboratory prepared H.C.G. (Prepn.

TABLE 16.

The effect of a previous injection of hyaluronidase on the in-vitro ovulation response of Xenopus laevis ovaries to H.C.G.

Hyaluronidase injected (I.U.)	H.C.G. added to ovary bath (I.U.)	Number of ovaries	
		ovulated	used
1,500	nil	0	1
1,500	87·5	0	1
1,500	175	0	1
1,500	350	0	1
1,500	700	2	2
3,000	nil	0	1
3,000	87·5	0	1
3,000	175	1	1
3,000	350	0	1
3,000	700	2	2

TABLE 17.

The effect of a previous injection of P.M.S. on the in-vitro ovulation response of Xenopus ovaries to H.C.G.

P.M.S. (I.U.)	H.C.G. (I.U.)	Number of ovaries	
		ovulated	used
nil	nil	0	1
20·62	nil	0	1
20·62	175	0	1
20·62	350	1	1
41·25	nil	0	1
41·25	41·25	0	1
41·25	82·5	0	1
41·25	175	1	2
41·25	350	2	5
82·5	nil	0	1
82·5	175	0	1
82·5	350	1	1
175	nil	0	1
175	175	0	1
175	350	0	1
350	nil	1	1
350	350	1	1

A) and a more purified preparation (Prepn. B). These preparations contained 70 and 500 I.U. per mg. respectively. Two pairs of ovaries were used, one of each pair was put in Xenopus saline containing Prepn. A, and the other into saline containing an equal number of I.U. of Prepn. B. Two dose levels were used. The only ovulation was in response to Prepn. A. (Table 18), and it therefore seems unlikely that impurities in H.C.G. inhibit in-vitro ovulation.

The dilution factor was next considered as a possible explanation for the different amounts of H.C.G. needed to cause ovulation when injected in-vivo and when added to the saline around the excised ovary. Gonadotrophin injected into the dorsal lymph sac passes quickly into the vascular system and is diluted by the volume of body fluids. The volume of these is likely to be less than that of the Xenopus saline used in-vitro. The next experiment was designed to investigate the effect of dilution in the ovary bath. The doses of H.C.G. administered to excised ovaries were each made up in 0·1 ml. Xenopus saline and were injected into each ovary which was put into 40 ml. Xenopus saline as usual. To prevent loss of injected fluid, each ovary was excised together with its attached kidney, and the injection was made through the kidney. The dose which caused ovulation in 4 out of 5 ovaries was 1/5th of the lowest amount found to cause

TABLE 18.

In-vitro ovulation of Xenopus laevis ovaries in response to preparations of H.C.G. with different activities.

Dose of H.C.G. (I.U.)		Number of ovaries	
Prepn. A.	Prepn. B.	ovulated	used
320		0	1
	320	0	1
640		1	1
	640	0	1

Preparation A contained 70 I.U. H.C.G. per mg.

Preparation B contained 500 I.U. H.C.G. per mg.

ovulation when put into the saline surrounding the ovaries (Table 19). This dose is similar to the M.E.D. 50 for H.C.G. injected in-vivo; it indicates that more H.C.G. has to be added to the ovary bath than needs to be injected in-vivo because, in the ovary bath, it is diluted by the Xenopus saline.

The effect of steroids on the excised ovary.

It has been suggested that, in the Anura, gonadotrophins alone do not bring about ovulation. I believe there is evidence that their effect is to stimulate the secretion of an ovarian steroid which causes the ova to be expelled. Some support to this hypothesis is provided by reports of ovulation in-vitro in response to added steroids. (Shapiro and Zwarenstein, 1937; Zwarenstein, 1937; Burgers and Zwarenstein, 1955; de Corral, 1959; Burgers and Li, 1960; Wright, 1961; Edgren and Carter, 1961, 1963; Chang and Tsaung, 1963; Ying-tien, 1963).

In the next experiment, the in-vitro activity of a number of steroids was investigated (Table 20). It will be seen that ovulation was stimulated by microgramme amounts of progesterone, methyl testosterone, hydrocortisone, oestrone and oestradiol. These results support the hypothesis that a steroid, secreted within the ovary, causes ovulation.

In the next experiment, the in-vitro activity of extracts of Xenopus ovaries was investigated. Ovaries

TABLE 19.

In-vitro ovulation of Xenopus laevis ovaries in response to H.C.G. injected directly into the excised ovary.

H.C.G. (I.U.)	Number of ovaries	
	ovulated	used
nil	0	1
4·375	0	1
8·75	0	1
17·5	0	1
35	4	5
70	2	3

TABLE 20.

In-vitro ovulation of Xenopus laevis ovaries in response to some steroids.

Dose of steroid	Number of ovaries which ovulated in response to each steroid					
	progesterone	methyl testosterone	oestradiol	oestrone	hydrocortisone	cortisone acetate
20 mg.	-	-	-	-	-	1(1)
16 mg.	-	4(4)	-	-	-	-
8 mg.	-	4(4)	1(1)	-	1(1)	1(1)
4 mg.	-	4(4)	-	-	-	-
2 mg.	-	3(3)	-	-	-	-
1 mg.	-	4(5)	-	-	-	-
0·5 mg.	-	3(3)	-	-	-	-
0·25 mg.	-	5(6)	-	-	-	-
128 µg.	1(1)	3(3)	1(2)	-	1(1)	-
64 µg.	1(1)	6(6)	-	-	1(1)	-
32 µg.	1(1)	2(2)	-	-	1(1)	-
16 µg.	1(1)	3(3)	-	-	-	-
8 µg.	2(3)	2(2)	0(1)	-	-	-
4 µg.	1(1)	2(2)	-	-	-	-
2 µg.	3(3)	4(4)	0(2)	2(2)	1(1)	-
1 µg.	2(2)	4(4)	2(2)	-	1(1)	-
0·5 µg.	1(2)	1(2)	3(4)	2(2)	-	-
0·25 µg.	1(1)	-	3(4)	-	-	-
0·125 µg.	0(1)	-	0(1)	0(2)	-	-

The figures in brackets indicate the number of ovaries used.

which had been stored in acetone for 2 years were extracted by the method of Dean and Chester Jones (1959). Two fractions were obtained; one which was expected to contain any progesterone in the ovaries (Fraction P), and the other any oestrogens present (Fraction O). Portions of the oily extracts were emulsified with Xenopus saline using Clensel D as the emulsifying agent. Control ovaries were set up in Xenopus saline with only the emulsifying agent added (Table 21). Each of the 3 ovaries to which Fraction P was administered ovulated, none of those to which Fraction O was added ovulated (Table 21). This result indicates the presence of a steroid in Xenopus ovaries which will directly stimulate ovulation and which is probably chemically similar to progesterone. Dean and Chester Jones remark on the difficulty of extracting oestrogen so it is possible that no oestrogens were contained in Fraction O. However no evidence of oestrogen or progesterone could be demonstrated in another extract using T.L.C. techniques.

The effect of A.C.T.H. on the excised ovary.

The stimulation of gloving which resulted from the injection of A.C.T.H. into the castrated and adenohypophysectomised male suggested that it caused the adrenal cortical tissue to secrete an androgenic steroid. The next experiment was designed to find out if such a steroid would cause ovulation in the excised ovary. Pairs of

TABLE 21.

In-vitro ovulation of Xenopus laevis ovaries in response to extracts of Xenopus ovaries

Dose of extract (≡ wet weight of ovary in mg.)	Fraction P		Fraction O	
	Number of ovaries		Number of ovaries	
	ovulated	used	ovulated	used
18·4	1	1	0	1
9·2	1	1	0	1
4·6	1	1	0	1
nil	0	5		

Fraction P = that fraction expected to contain any progesterone.

Fraction O = that fraction expected to contain any oestrogen.

ovaries were removed from 3 toads, the kidney and adrenal tissue was removed with each left ovary, and left attached to it. An equal dose of A.C.T.H. was administered to each ovary of a pair; 3 other ovaries received A.C.T.H. and 1 ovary was put in plain Xenopus saline (Table 22). It will be seen that A.C.T.H. caused ovulation in the presence and absence of adrenal tissue. It seems likely that its activity is similar to that of H.C.G. but it is difficult to find an explanation for the lack of in-vivo ovulation following injection of A.C.T.H. It is possible that exogenous A.C.T.H. has an inhibitory effect in-vivo which balances its direct stimulation of the ovary.

The time taken for injected H.C.G. to initiate ovulation.

This experiment was done to find out how long after injection of H.C.G. the process of ovulation is irreversibly initiated. Two female toads were injected with 70 I.U. H.C.G. and one of their ovaries removed under anaesthesia 4 hours and 6 hours, respectively, after injection, and set up in Xenopus saline. The other ovary was left in the toad to check that the ovaries were sensitive to this dose of H.C.G. Ovulation in-vitro only occurred in the ovary removed 6 hours after injection, but both the ovaries left in the toads ovulated. The experiment was then repeated with ovaries removed at half-hourly intervals; one ovary from each of 2 toads being removed at each $\frac{1}{2}$ hour interval after injection (Table 23).

TABLE 22

In-vitro ovulation of Xenopus laevis ovaries in response to A.C.T.H.

Kidney and adrenal tissue (x = presence) (- = absence)	Dose of A.C.T.H. (I.U.)	Number of ovaries	
		ovulated	used
-	nil	0	1
-	5	0	3
* (-	5	1	1
* (x	5	1	1
* (-	10	1	1
* (x	10	1	1
* (-	15	1	1
* (x	15	1	1

* indicates a pair of ovaries from the same toad.

TABLE 23.

In-vitro and in-vivo ovulation of Xenopus laevis ovaries in response to 70 I.U. H.C.G. injected at different intervals before unilateral ovariectomy.

Interval between injection and excision of one ovary (hours)	Number of toads injected	Number of ovulations of:	
		the excised ovary in-vitro	the unexcised ovary in-vivo
3·0	2	0	2
3·5	2	2	2
4·0	2	1	1
4·5	2	2	2
5·0	2	2	2

All the ovaries removed at 3·5 hours or more after injection ovulated, except for one which was shown not to be sensitive by the failure of its pair to ovulate in-vivo. This result showed that ovulation is irreversibly initiated within 3·5 hours of injection of an ovulatory dose of H.C.G.

The activity of injected steroid in causing in-vivo ovulation and oviposition

The previous in-vitro experiments have shown that different types of steroids will cause ovulation of the excised ovary. The following experiment was done to find out if injected steroids will cause ovulation in X. laevis. Various steroids were injected at different dose levels (Table 24). It will be seen that ovulation and oviposition was induced by methyl testosterone, progesterone, prednisolone, cortisone acetate and hydrocortisone but not by oestradiol or D.O.C.A. These results support the hypothesis that ovulation may be caused by a steroid secreted by, or synthesised within the ovary under gonadotrophic stimulation. The number of different steroids which will cause ovulation in-vivo and in-vitro makes it seem likely that these exogenous steroids may be acting as intermediates in the synthesis by the ovary of the natural ovulatory steroid. It is difficult to explain why low doses of oestradiol cause ovulation of the excised ovary but not oviposition in the intact animal. Exogenous

TABLE 24.

The ovulation and oviposition responses of female Xenopus laevis injected with steroids and with A.C.T.H.

Substance injected	Number of toads which oviposited					
	Dose injected (mg.)					
	0·3125	0·625	1·25	2·5	5·0	10·0
methyl testosterone	0 (4)	2 (4)	4 (4)	-	-	-
progesterone	-	2 (5)	0 (5)	-	-	-
oestradiol	-	0 (5)	0 (5)	died (4)	-	-
prednisolone	-	0(20)	12(30)	8(10)	-	-
cortisone acetate	-	-	-	-	0 (5)	5(10)
hydrocortisone	-	-	5(20)	12(20)	12(20)	-
D.O.C.A.	0 (3)					
30 I.U. A.C.T.H.	0 (3)					

The figures in brackets indicate the number of toads injected.

oestradiol is toxic in the intact animal; when toads were injected with higher doses, fluid accumulated in their lymph sacs and they died. No ovulation resulted when 5 toads were each given 7 injections of 1 mg. oestradiol over 3 weeks.

The effect of simultaneous injection of a corticosteroid on the ovulation response to H.C.G.

It has been suggested that cortisone assists the action of gonadotrophins in causing ovulation in R. pipiens (Chang and Witschi, 1957). In the following experiments, the effects of cortisone acetate and hydrocortisone, respectively, on the ovulation response to H.C.G. were investigated. Various doses of these 2 steroids were injected, alone and together with H.C.G. (Tables 25 and 26). With H.C.G., the effect of both these steroids appeared to be additive.

The effect of hydrocortisone on the gonadotrophic activity of the pituitary.

When "priming" toads which had been injected with hydrocortisone 10 days previously, it was noticed that these toads seemed to have a reduced sensitivity to injected H.C.G. To investigate this, 20 toads which had ovulated 10 days previously to 70 I.U. H.C.G. were divided into 2 weight-matched groups; each toad of one group was injected with 6 mg. hydrocortisone. Eleven days later each toad of both groups was injected with 70 I.U. H.C.G.

TABLE 25.

The effect of simultaneous injection of cortisone acetate on the ovulation and oviposition response of female Xenopus laevis to injection of H.C.G.

		Percentages of toads which oviposited		
		mg. cortisone acetate injected		
		nil	5·0	10·0
	nil	-	0 (5)	50 (10)
I.U.	8·75	-	37(19)	-
H.C.G.	17·5	0 (18)	65(27)	-
injected	35·0	40 (10)	80(10)	-
	70·0	80 (30)	100(10)	-
	175·0	90 (10)	-	-

The figures in brackets indicate the number of toads injected.

TABLE 26.

The effect of simultaneous injection of hydrocortisone on the ovulation and oviposition response of female Xenopus laevis to injection of H.C.G.

		Percentages of toads which oviposited			
		mg. hydrocortisone injected			
		nil	1·25	2·5	5·0
	nil	-	25 (20)	60 (20)	60 (20)
I.U.	17·5	0 (10)	50 (10)	86 (7)	94 (18)
H.C.G.	35·0	50 (10)	-	-	-
injected	70·0	100 (10)	-	-	-

The figures in brackets indicate the number of toads injected.

Table 27 shows the result; the difference in ovulation response to H.C.G. after injection of hydrocortisone appears to confirm the subjective impression, but the difference is not significant. By an oversight, no assays of gonadotrophic activity were performed on partes distales from female Xenopus injected with hydrocortisone. However, partes distales from males injected with hydrocortisone were found to have a reduced gonadotrophic activity (Table 10, page 125). These 2 pieces of evidence, taken together, indicate that exogenous hydrocortisone inhibits gonadotrophin secretion (or synthesis) by the adenohypophysis.

The activity of injected A.C.T.H.

The gloving activity of injected A.C.T.H. in the male (Figure 22, page 109) suggested testing it for ovulatory activity in female X. laevis. Four toads were each injected with 30 I.U. A.C.T.H. No ovulation resulted. The same toads were then given 7 more injections of the same dose at 4 day intervals without oviposition resulting. Three toads were injected with 300 I.U. each and 3 toads with 3000 I.U. each without causing oviposition. These results indicate that exogenous A.C.T.H. does not cause ovulation in X. laevis.

Cloacal hyperaemia

When female X. laevis ovulate, their cloacal labia become red and swollen as a result of hyperaemia of the

TABLE 27.

The effect of a previous injection of hydrocortisone on the ovulation and oviposition response of female Xenopus laevis to injected H.C.G.

Date of injection	Ten control toads		Ten experimental toads	
	Dose and substance	Number oviposited	Dose and substance	Number oviposited
6/1/60	1 ml. distilled water	0	6 mg. hydrocortisone	7
14/1/60	70 I.U. H.C.G.	8	70 I.U. H.C.G.	5

blood vessels (Plates IX and X). This cloacal hyperaemia occurs when toads ovulate in response to injected H.C.G.

The following experiment was designed to investigate the ratios of ovulation and cloacal hyperaemia inducing activities of 2 gonadotrophins. H.C.G. was used as a gonadotrophin with almost entirely luteinizing activity and P.M.S. was used because it has mainly follicle-stimulating activity. Four groups of females were injected with a range of doses of P.M.S. and 3 groups with a range of doses of H.C.G. (Table 28). The numbers of toads ovulating and with cloacal hyperaemia were recorded on each subsequent day until the figure for each was zero (Table 28). P.M.S. tended to be more effective in causing cloacal hyperaemia, and H.C.G. more effective in stimulating ovulation.

To find out if these gonadotrophins have a direct or indirect action on the cloacal labia, 10 ovariectomised females were each injected with 800 I.U. P.M.S. and another 10 ovariectomised toads were injected with 240 I.U. H.C.G. No cloacal hyperaemia resulted in either group. This indicated that cloacal hyperaemia is produced in normal females by an ovarian hormone secreted as a result of gonadotrophic stimulation.

These results show that, whereas ovulation in X. laevis is stimulated by a luteinizing type of gonadotrophin, a follicle stimulating type is more effective in

PLATE IX.

Cloacal labia of a female Xenopus in the non-hyperaemic state.

PLATE X.

Hyperaemic cloacal labia of a female Xenopus laevis ovulating in response to injected H.C.G.

TABLE 28.

The ovulation and cloacal hyperaemic responses of groups of 10 female Xenopus laevis injected with P.M.S. and H.C.G.

Toads given a single injection of P.M.S.					Toads given a single injection of H.C.G.				
Dose (I.U.)	Days after injection	Number of toads		CH/O	Dose (I.U.)	Days after injection	Number of toads		CH/O
		CH*	O*				CH*	O*	
240	1	2	0	2/0	80	1	9	9	9/9
	2	3	0	3/0		2	1	8	1/8
	3	2	0	2/0		3	0	5	0/5
	4	0	0	-		4	0	1	0/1
480	1	5	2	5/2		5	0	0	-
	2	5	5	5/5	240	1	10	10	10/10
	3	4	3	4/3		2	1	9	1/9
	4	4	3	4/3		3	1	9	1/9
	5	0	0	-		4	0	4	0/4
800	1	9	8	9/8		5	0	4	0/4
	2	8	10	8/10		6	0	2	0/2
	3	5	9	5/9		7	0	1	0/1
	4	3	2	3/2		8	0	0	-
	5	1	2	1/2	720	1	10	10	10/10
	6	1	0	1/0		2	10	10	10/10
	7	0	0	-		3	8	9	8/9
1,120	1	10	10	10/10		4	6	9	6/9
	2	10	10	10/10		5	2	9	2/9
	3	9	9	9/10		6	0	3	0/3
	4	8	4	8/4		7	0	0	-
	5	5	4	5/4					
	6	4	0	4/0					
	7	1	0	1/0					

* CH = with hyperaemic cloacal labia

O = ovulating and ovipositing

stimulating the secretion of the ovarian steroid which induces cloacal hyperaemia.

In an attempt to discover the steroid responsible for causing cloacal hyperaemia 5 intact females and 5 toads which had been ovariectomised 2 months previously were each given 10 injections of 1 mg. oestradiol over 10 weeks. No cloacal hyperaemia resulted. The experiment was repeated on similar groups with the same number of injections of 1 mg. progesterone. No cloacal hyperaemia resulted.

The next experiment was designed to find out if an endogenous adrenocorticosteroid might stimulate cloacal hyperaemia.

Four ungloved intact females and four ungloved females which had been ovariectomised 2 months previously were each given 10 injections, each containing 30 I.U. A.C.T.H., over 5 weeks. No cloacal hyperaemia occurred. Three of the intact toads were then each given a single injection of 300 I.U. A.C.T.H. followed by another of 3,000 I.U. 4 days later. No cloacal hyperaemia was observed. (None of the toads ovulated at any time during the experiment.*) It would appear that an endogenous adrenocorticosteroid is not involved in the stimulation of cloacal hyperaemia.

---

* This aspect of the part of this experiment in which intact females were injected with A.C.T.H. is reported on page 154.

DISCUSSION

According to Bellerby (1938), nutritional changes are one of several factors which affect the sexual cycle of X. laevis under natural conditions. In some of my experiments it was necessary to keep toads in jars for several weeks. When kept in jars, toads eat less food than when they are in tanks. It was found that, over a period of thirteen weeks, less frequent feeding reduced the amount of liver eaten but this had no effect on gloving or the spermiation response of male X. laevis to injected H.C.G. This observation is important because it eliminates reduced food intake as a factor in gloving and spermiation experiments in which toads have to be kept in jars for several weeks. It is known that the ovaries of X. laevis will regress if an adequate food supply is not available (Alexander and Bellerby, 1935, 1938; Bellerby, 1938; Landgrebe, 1939). This difference between the male and female is probably due to the large amount of protein needed for vitellogenesis.

Evidence that the proximity of other animals has an effect on the sexual characters does not appear to have been previously reported in an Amphibian. My finding that both gloving and spermiation of male X. laevis is inhibited by the proximity of other Xenopus of either sex and by the presence of goldfish is therefore significant.

It could account for the regression of the ovaries of female Xenopus which were kept in overcrowded conditions by Alexander and Bellerby (1938). The observation that adenohypophysectomy removes the difference between the spermiation response of toads isolated in jars and toads kept together in tanks suggests that the difference in intact toads is due to an effect on the adenohypophysis. It seems likely that close proximity of other animals inhibits adenohypophyseal gonadotrophin secretion. Bles (1905) observed that simulated rainfall and increased water temperature will cause oviposition in Xenopus. This suggests that changes in the external environment may cause an alteration in the level of hypophyseal gonadotrophin secretion. It is possible that the ovary becomes more sensitive to endogenous gonadotrophin.

My experiments have shown that water volume per toad does not itself affect gloving or spermiation. It therefore seems likely that the cessation of ovarian activity, observed by Alexander and Bellerby (1938), may not be directly due to changes in water volume, as suggested by Bellerby (1938). It is probable that changes in water volume have an indirect effect by altering the concentration of Xenopus and other animals in the ponds.

My finding that gloving is maintained in castrated males, and appears in normal females indicates the involve-

ment of the adrenal cortex in the maintenance of gloving in X. laevis. This is supported by the stimulation of gloving in castrated and adenohypophysectomised males by injections of hydrocortisone, and also by exogenous gonadotrophin and corticotrophin. It is possible that hydrocortisone is more similar to the natural androgen secreted by both the testes and adrenals of X. laevis, as it is more potent than methyl testosterone in restoring gloving in the castrated and adenohypophysectomised animal. Alterations in the secretion of hydrocortisone may, at least partly, explain how rainfall influences gloving, and may reinforce the effect of alterations in the population density.

Hydrocortisone has been reported to have a diuretic activity in the Amphibia (Jenkin, 1962). If the amount secreted is influenced by the osmotic pressure of the water in which Xenopus live, it seems likely that less will be secreted as the ponds dry up and the concentration of solutes rises. This would explain the regression of gloving which follows the normal seasonal decrease in rainfall. Correspondingly, it is suggested that, when the ponds fill up and the osmotic pressure of the water falls, Xenopus secrete more hydrocortisone. This would stimulate gloving as well as causing a diuresis. This could explain the slight increase in the extent of gloving which occurs after the injection of distilled water. It

is interesting that Xenopus has been reported to have a different type of water regulation from that of other Anura (except, presumably, other species of Aglossa). Xenopus cannot increase their water uptake after dehydration and show virtually no antidiuretic response to neurohypophyseal extracts (Barrington, 1963).

I suggest that the adrenal cortical tissue of Xenopus may contain cells which secrete a steroid - probably hydrocortisone - which is androgenic in Xenopus. It is possible that these cells are similar to the Stilling cells of some other Anurans. No evidence of their presence in Xenopus has been published. Chester Jones (1956) states that the nature of Stilling cells is still problematical. However, it seems significant that they are plentiful in R. esculenta, in which spermatogenesis is a continuous process, but absent from R. temporaria, in which spermatogenesis does not take place in the autumn and winter (van Oordt and van Oordt, 1955; Chester Jones, 1956). This indicates a connection between sexual activity and the presence of Stilling cells. Stilling cells are dependent upon pituitary stimulation as they regress after hypophysectomy in R. esculenta (Sluiter, van Oordt and Mighorst, 1949). This is compatible with my finding that gloving in castrated male X. laevis completely disappears when they are also adenohypophysectomised. My observation that gloving is

maintained in castrated and adenohypophysectomised male X. laevis by injections of A.C.T.H. is compatible with the responsiveness of Stilling cells to A.C.T.H. (Gorbman, 1964). This author states that the function of these cells is completely unknown but suggests they may serve as part of an integrated endocrine mechanism.

The ineffectiveness of A.C.T.H. in stimulating cloacal hyperaemia in female X. laevis would suggest that the adrenal cortical tissue is not directly involved in the secretion of the endogenous steroid responsible for stimulation of cloacal hyperaemia. However, the development of a certain degree of gloving in intact and ovariectomised females which were injected with A.C.T.H. indicates that the female adrenal tissue is able to secrete an androgen.

The only other report which indicates that male Anuran secondary sex characters may have an extra-testicular control is that of Moszkowska (1932) who found that injections of pituitary extract stimulated development of the thumb pads in castrated male B. bombina. Lofts (1964) quoted this as evidence of a possible direct action of the pituitary upon the secondary sex characters. I suggest that it indicates that exogenous pituitary extract stimulates adrenocortical secretion of an androgen. The androgenic activity of adrenal tissue cannot be directly tested by adrenalectomy in Xenopus as it is not only embedded in the kidneys but also in the walls of the

renal veins and the posterior vena cava (Zwarenstein and Schrire, 1932).

The testicular control of gloving in X. laevis is shown by the regression of gloving after castration, and confirmed by the redevelopment of extensive gloving in castrates after injection of testosterone. Berk (1939) was probably unsuccessful in stimulating gloving in castrated Xenopus because he injected lower doses of testosterone than those used in this investigation.

It seems to be generally agreed that the interstitial cells of the testes are responsible for androgen secretion in Amphibia. Many workers have correlated the development of the nuptial characters in adult male Amphibia with an increase in the number of interstitial cells, and the first appearance of these cells in larvae has been correlated with the first development of male secondary sex characters (Aron, 1924, 1926; Smith, 1938; Glass and Rugh, 1944; Iwasawa and Asai, 1959; Lofts, 1964).

The adenohypophyseal control of androgen secretion in X. laevis is indicated by my finding that gloving disappears after adenohypophysectomy but may be re-induced by injecting gonadotrophin. It is not yet known if there are two gonadotrophic hormones secreted by the Amphibian adenohypophysis. The evidence for one hormone which stimulates gametogenesis and another which stimulates the interstitial cells is not conclusive and is largely

histological. van Oordt (1961) inferred that the β-cells of the R. temporaria pars distalis secrete F.S.H. because castration caused hyperactivity of these cells and the administration of testosterone caused a reduction of their secretory activity. A better correlation between the γ-cells and L.H. secretion was shown by the correlation between regression of secondary sex characters and regression of γ-cells in frogs kept at high temperatures. An indication that the γ-cells may secrete L.H. is provided by their poor development in laboratory-reared Xenopus (van Oordt, 1963). X. laevis which are kept in the laboratory do not usually ovulate spontaneously and a luteinizing type of gonadotrophin has to be injected to cause ovulation. It therefore seems likely that natural ovulation is caused by L.H. secreted by the γ-cells of the adenohypophysis. When adenohypophysectomised male X. laevis are injected with doses of P.M.S. and H.C.G. of equal spermatokinetic activity, the gloves are retained longer in the animals receiving H.C.G. In female X. laevis, injections of P.M.S. have a greater effect in stimulating cloacal hyperaemia than in causing ovulation whereas the activities of H.C.G. are the other way round. From these results it would seem that there may be 2 gonadotrophins secreted by the adenohypophysis of Xenopus, F.S.H. which stimulates spermatogenesis in

the male and cloacal hyperaemia in the female, and L.H. which causes ovulation in the female and stimulates androgen secretion in the male. As exogenous P.M.S. has a greater activity in causing spermiation than in stimulating gloving it seems likely that F.S.H. is responsible for initiating spermiation in X. laevis. However, the inability of exogenous F.S.H. to cause spermiation in other species of Anura suggests that Xenopus may be unique in this respect.

Ovulation in X. laevis is only stimulated by a gonadotrophin with luteinizing activity whereas spermiation can be stimulated by either type of gonadotrophin. The abnormal ratio of M.E.D. 50 for spermiation to the M.E.D. 50 for ovulation in adenohypophyses from 2 castrated male Xenopus indicates that the Xenopus pars distalis secretes 2 gonadotrophins and that the testes specifically inhibit L.H. secretion. This is supported by the very low ovulation-inducing activity of adenohypophyses from female toads, except for those from 2 toads, one of which was incompletely ovariectomised and the other had atrophic ovaries. This suggests that ovariectomy removes a specific inhibition of L.H. secretion.

The observations of Berk and Shapiro (1939) suggested that progesterone might play some part in the

induction and maintenance of cloacal hyperaemia in female Xenopus. In my experiments, I was unable to produce hyperaemia of the cloacal labia with progesterone. The difference may be due to the breeding condition of the animals. The toads used in this investigation had been in the laboratory for at least a year and were kept in conditions under which they do not spontaneously ovulate. Those used by Berk and Shapiro were "freshly brought in from the ponds" and none had been in their laboratory for more than 2 weeks when injected. They do not state at what time of year their toads were collected, so it is possible that the cloacal labia were already about to become hyperaemic, as, in their natural habitat, Xenopus spawn several times between July and December (Hey, 1946). It is unfortunate that the experiments of Berk and Shapiro did not include the appropriate controls.

Normal female X. laevis readily ovulate when injected with H.C.G. In direct contrast the excised ovary is refractory to gonadotrophin but sensitive to very much lower doses of corticosteroids than are necessary to cause ovulation when injected, and also ovulates in response to steroids which do not cause ovulation when injected.

Following the method of Wright (1961a, b), I investigated in-vitro ovulation in X. laevis. Pieces of excised ovary were placed in Petri dishes containing Xenopus saline. This method had several disadvantages. Ova were sometimes liberated from the cut edges of the ovarian fragments placed in plain Xenopus saline. This indicated a possibility of "false positive" reactions. Because the pieces of ovary lay on the bottom of the dishes, ovulation from the lower parts was impeded, and the dishes had to be agitated to observe any shed ova. This practice added to the risk of freeing unovulated ova from the cut edges. The apparatus designed for subsequent experiments was an important advance as it allowed me to use whole ovaries and to carefully control the temperature and other conditions of the experiments.

With the excised ovaries suspended in Xenopus saline, ovulation could be observed as it occurred. The validity of the in-vitro results was further improved by aeration, sterilization of the apparatus and saline and by the addition of penicillin to the ovary baths. Each of these techniques extended the life of the excised ovaries; these appeared

healthy after several days. This eliminated ovarian decomposition as a factor causing liberation of ova within the 24 hour period used as a criterion of normal ovulation.

Using these techniques, excised Xenopus ovaries were found not to ovulate to less than half a Xenopus A.L.P. in 40 ml. of Xenopus saline. This concentration is 64 times greater than that of frog A.L.P. to which R. pipiens ovaries were reported to ovulate in-vitro (Wright, 1945), but Wright used Holtfreter's solution which is hypotonic to adult Anuran tissues. As I have found that hypotonicity of the surrounding fluid causes ovulation of the excised Xenopus ovary, it seems likely that the same effect assisted the effect of frog A.L.P. in Wright's experiments. Although the lowest dose of H.C.G. which caused in-vitro ovulation of Xenopus ovaries was 175 I.U., ovulation was stimulated when 35 I.U. was injected directly into the excised ovary. This dose is similar to the M.E.D. 50 for in-vivo ovulation in response to injected H.C.G. (Hobson, 1952).

The relatively greater sensitivity of the excised Xenopus ovary to steroids compared with gonadotrophins supports the hypothesis that injected gonadotrophin stimulates ovulation by causing the secretion of an ovarian steroid which initiates ovulation. My finding that in-vitro ovulation of Xenopus ovaries can be stimulated with

progesterone and methyl testosterone agrees with reports for other species of Anura (Langan, 1941; Wright, 1961a; Edgren and Carter, 1961, 1963; Ying-tien, 1963; Chang and Tsaung, 1963), but differs from the inactivity of progesterone and nor-ethinyl testosterone on the excised ovaries of B. arenarum reported by de Corral (1959), although the latter author did not state the doses he used.

The induction of ovulation in excised Anuran ovaries with an oestrogen is the first reported. Oestrone and oestradiol have been found inactive in stimulating in-vitro ovulation of R. pipiens ovaries (Langan, 1941; Wright, 1961a) and Personen and Rapola (1962) stated categorically that oestrogens never have in-vitro ovulatory activity. Oestriol has been reported to inhibit the in-vitro activity of progesterone on R. pipiens ovaries (Edgren and Carter, 1963) but Wright, (1961b) found that oestrone and oestradiol benzoate potentiate the in-vitro activity of progesterone but inhibit the ovulatory response of R. pipiens ovaries to A.L.P. extracts of the same species.

Although Chang and Witschi, (1957) believe that a corticosteroid has only a "last step" effect on ovulation in R. pipiens, my results indicate that ovulation in Xenopus is probably induced by a steroid. This is supported by the in-vitro ovulation caused by the extract of Xenopus ovaries which I made.

It is difficult to explain the activity of A.C.T.H. in stimulating ovulation of the excised ovary and its

failure to produce ovulation when injected into the toad. It may be that the ovary secretes an ovulatory steroid in response to A.C.T.H. but that, when injected into the intact toad, A.C.T.H. has some other action which counteracts its stimulatory effect upon the ovary. In B. arenarum, A.C.T.H. has been reported to be ineffective in causing ovulation in-vivo or in-vitro (de Corral, 1959).

The possibility that hyaluronidase might assist in the mechanism of ovulation was suggested to me by van Oordt's hypothesis that this enzyme is involved in spermiation. van Oordt (1962) suggested that hyaluronidase, by hydrolysing hyaluronic acid, increases the colloid osmotic pressure in the testis tubules of male Rana which causes an uptake of water and the liberation of sperm. It seemed to me that the expulsion of the ovum from its follicle might be caused by a similar mechanism. However, my experiments did not support this hypothesis. It may be that, to cause an osmotic pressure gradient, the hyaluronidase must be liberated only within the follicle.

The secretion of steroids from the gonads of X. laevis is indicated by the effects of gonadectomy, and the injection of steroids on the gonadotrophin content of the pars distalis indicates that the gonads of Xenopus secrete steroids. The partes distales of male toads which had been castrated for a period of 2 years or more were about

28 times more active than those from normal toads in terms of spermiation-inducing activity. The gonadotrophic activity of the pars distalis is reduced in those toads which have been injected with steroid. Although testosterone had little effect, the activity of 2 pooled adenohypophyses from males injected with hydrocortisone was so low that no spermiation response was obtained from as much as 2/5ths of a pars distalis.

The results obtained did not demonstrate any significant difference between the gonadotrophic activities of partes distales from male Xenopus with gloving at various stages. However, the pooled partes distales from 5 fully gloved males were just over twice as active in stimulating spermiation as the mean activity of those from 10 toads with only their hands gloved. A greater correlation between gonadotrophic activity of the pars distalis and the state of gloving of the toad from which it was taken might be shown if the partes distales could be assayed using the induction of gloving in adenohypophysectomised toads as the response. This was precluded by the number of partes distales which would be needed.

Although the gonadotrophic activity of partes distales from female Xenopus is, in general, greater than that of those from males, the effect of ovariectomy in the female is the same as that of castration in the male - an increase in gonadotrophic activity expressed per fraction

of the pars distalis. The gonadotrophic activity of adenohypophyses from female Xenopus which had been injected with oestradiol was not, however, less than those from normal uninjected females.

The results of this investigation have shown that in Xenopus, as in the Mammalia, the adenohypophysis regulates gonadal gametogenesis and also the gonadal and adrenocortical control of secondary sex characters. The activity of the adenohypophysis has been found to be regulated by the gonads and by external factors.

REFERENCES.

Adams, A.E. (1940). Amer. J. Anat. 66, 235.

Adams, A.E. (1950). J. exp. Zool. 113, 463.

Alexander, S.S. and Bellerby, C.W. (1935). J. exp. Biol. 12, 306.

Alexander, S.S. and Bellerby, C.W. (1938). J. exp. Biol. 15, 74.

de Allende I.L.C. (1939). C.R. Soc. Biol., Paris, 130, 163.

de Allende I.L.C. and Orias, O. (1955). Acta. Physiol. latinoam. 5, 57.

Aron, M. (1924). Archs. Biol., Paris. 34, 1.

Aron, M. (1926). Archs. Biol., Paris. 36, 3.

Astrada, J.J. (1962). Acta. Physiol. latinoam. 12, 243.

Atwell, W.J. (1941). Amer. J. Anat. 68, 191.

Atz, E.H. and Pickford, G.E. (1954). Zoologica, N.Y. 39, 117.

Baglioni, S. (1911). Zbl. Physiol. 25, 233.

Ball, J.N. and Cowen,P.N. (1959). Nature. Lond. 184, 370.

Barrington, E.J.W. (1963). An introduction to General and Comparative Endocrinology. Clarendon Press.

Basu, S., Bern, H.A. and Chan, H. (1965). Anat. Rec. 151, 441.

Bellerby, C.W. (1938). J. exp. Biol., 15, 82.

Bellerby, C.W. and Hogben, L. (1938). J. exp. Biol. 15, 91.

Berk, L. (1938). S. Afr. J. med. Sci. 3, 72.

Berk, L. (1939). S. Afr. J. med. Sci. 4, 47.

Berk, L. and Shapiro, H. (1939). S. Afr. J. med. Sci. 4, (Suppl.) 13.

Bhaduri, J.L. (1951). Proc. 38th Indian Sci. Congr. (sect. Zool. and Entomol.) 1.

Blair, A.P. (1946). J. exp. Zool. 103, 365.

Bles, E.J. (1901). Proc. Camb. phil. Soc. 2, 220.

Bles, E.J. (1905). Trans. roy. Soc. Edinb. 16, 789.

Bresca, G. (1910). Arch. f. EntwMech. Org. 29, 403.

Burgers, A.C.J. and Li, C.L.I. (1960). Endocrinology. 66, 255.

Burgers, A.C.J. and Zwarenstein, H. (1955). Nature. Lond. 175, 687.

Burgos, M.H. (1949). Rev. Soc. argent. Biol. 25, 206.

Burgos, M.H. (1950a). C.R. Soc. Biol., Paris. 144, 420.

Burgos, M.H. (1950b). Rev. Soc. argent. Biol. 26, 359.

Burgos, M.H. (1951). C.R. Soc. Biol., Paris. 145, 444.

Burgos, M.H. (1952). C.R. Soc. Biol., Paris. 146, 609.

Burgos, M.H. (1955). J. Morph. 96, 283.

Burgos, M.H. and Ladman, A.J. (1955). Proc. Soc. exp. Biol., N.Y. 88, 484.

Burgos, M.H. and Ladman, A.J. (1957). Endocrinology, 61, 20.

Burns, R.K. Jr. and Buyse, A. (1932). Anat. Rec. 51, 333.

Callard, I.P. and Leatham, J.H. (1966). Gen. comp. Endocr. 7, 80.

Chang, C.Y. and Witschi, E. (1957). Endocrinology. 61, 514.

Chang, C.Y. and Tsaung, L.T. (1963). Acta. Biol. exp. sin. 8, 478.

Charipper, H.A. and Martorano, H.A. (1948). Zoologica., N.Y. 33, 157.

Chester Jones, I. (1956). The Adrenal Cortex. Cambridge University Press.

Chieffi, G. and Lupo, Concetta. (1963). Gen. comp. Endocr. 3, 149.

Chieffi, G. and Lupo, Concetta. (1961). R.C. Accad. Lincei. 8 VIII 30, 399.

Christensen, K. (1931). Anat. Rec. 48, 241.

Cordier, R. (1953). Ann. Soc. Zool., Belg. 84, 5.

de Corral, J.M. (1959). C.R. Soc. Biol., Paris. 153, 493.

Creaser, C.W. and Gorbman, A. (1939). Quart. Rev. Biol. 14, 311.

Dale, E. (1962). Gen. comp. Endocr. 2, 171.

Dean, F.D. and Chester Jones, I. (1959). J. Endocrin. 18, 366.

Della-Corte, F. and Cosenza, L. (1965). Gen. comp. Endocr. 5, 679.

Dierickx, K. (1964a). Z. Zellforsch. 61, 920.

Dierickx, K. (1964b). Z. Zellforsch. 63, 938.

Dierickx, K. (1965). Z. Zellforsch. 66, 504.

Dierickx, K. (1966a). Naturwissenschaften. 53, 279.

Dierickx, K. (1966b). Naturwissenschaften. 53, 709.

Dodd, J.M. (1960). in Marshalls Physiology of Reproduction. Vol. 1. Pt. 2. p. 417. Ed. A.S. Parkes. Longmans.

Doerr-Schott, Jeannine. (1965). Gen. comp. Endocr. 5, 631.

Donahue, J.K. (1934). Proc. Soc. exp. Biol., N.Y. 31, 1166.

van Dongen, W.J., Ballieux, R.E., Geurson, H.J. and Offermans, T. (1960). Proc. K. Ned. Akad. Wet., Ser. C. 62, 320.

van Dongen, W.J., Jørgensen, C.B., Larsen, L.O., Rosenkilde, P., Lofts, B., and van Oordt, P.G.W.J., (1965). Gen. comp. Endocr. 6, 49.

van Dongen, W.J. and de Kort, E.J.M. (1959). Proc. K. Ned. Akad. Wet., Ser. C. 62, 320.

Edgren, R.A. and Carter, D.L. (1961). Gen. comp. Endocr. 1, 492.

Edgren, R.A. and Carter, D.L. (1963). Gen. comp. Endocr. 3, 526.

Edinger, D. (1913). Ztschr. allg. Physiol. 15, 15.

Galgano, M. (1931). Monit. zool. Ital. 42, 297.

Galgano, M. (1932). Monit. zool. Ital. 43, 156.

Galgano, M. (1934). Monit. zool. Ital. 45, (Suppl.) 82.

Galgano, M. (1935). Arch. ital. Anat. Embriol. 53, 511.

Galgano, M. (1936). Monit. zool. Ital. 46, 273.

Galgano, M. (1941). Monit. zool. Ital. 52, 233.

Galgano, M. (1947). Arch. zool. (ital.), Napoli. 32, 33.

Gallien,L. (1937). C.R. Acad. Sci., Paris. 205, 375.

Gallien, L. (1938). Bull. Biol. 72, 269.

Gallien, L. (1939). C.R. Soc. Acad. Sci., Paris. 208, 766.

Gallien, L. (1940). Bull. Biol. 74, 1.

Gallien, L. (1944). Bull. Biol. 78, 257.

Gallien, L. (1955). Mem. soc. Endocr. 4, 188.

Gallien, L. and le Foulgoc, M.Th.C. (1960). C.R. Acad. Sci. 251, 460.

Galli-Mainini, C. (1950). Rev. Soc. argent. Biol. 26, 166.

Galli-Mainini, C. (1951). C.R. Soc. Biol., Paris. 145, 131.

Gitlin, G. (1939). S. Afr. J. med. Sci. 4, (Suppl.) 41.

Gitlin, G. (1941). S. Afr. J. med. Sci. 6, 136.

Gitlin, G. (1947). S. Afr. J. med. Sci. 12, 141.

Giusti, L. and Houssay, B.A. (1923). C.R. Soc. Biol., Paris, 89, 739.

Giusti, L. and Houssay, B.A. (1924). C.R. Soc. Biol., Paris, 91, 313.

Glass, F.M. and Rugh, R. (1944). J. Morph. 74, 409.

Gorbman, A. (1964). Endocrinology of the Amphibia in "Physiology of the Amphibia". Ed. J.A. Moore, Acad. Press.

Grant, R. (1940). Anat. Rec. 78, (Suppl.) 86.

Greenberg, B. (1942). J. exp. Zool. 91, 435.

Greenblatt, R.B., Clark, S. and West, R.M. (1949). J. clin. Endocrin. 9, 668.

Harms, J.W. (1926). Korper und Keimsellen. Springer. Berlin.

Harris, G.W. (1955). Neural Control of the Pituitary Gland. Arnold.

Heilbrun, L.V.K., Daugherty, K. and Wilbur, K.M. (1939). Physiol. Zoöl. 12, 97.

Herring, P.T. (1913). Quart. J. exp. Physiol. 6, 73.

Hey, D. (1949). Trans. roy. Soc. S. Afr. 32, 45.

Hoar, W.S. (1966). in The Pituitary Gland vol. I. Ed. G.T. Harris and B.T. Donovan. Butterworths.

Hobson, B.M. (1952). Quart. J. exp. Physiol. 37, 191.

Hobson, B.M. (1955). Thesis. Edinburgh University.

Hobson, B.M. (1965). Acta endocr., Copenhagen. 49, 337.

Hobson, B.M. (1968). Personal communication.

Hobson, B.M. and Landgrebe, F.W. (1954). Quart. J. exp. Physiol. 39, 23.

Hogben, L. (1923). Quart. J. exp. Physiol. 13, 177.

Hogben, L. (1930). Proc. roy. Soc. S. Afr. March 19.

Hogben, L., Charles, Enid. and Slome, D. (1931). J. exp. Biol. 8, 345.

Houssay, B.A. (1949). Quart. Rev. Biol. 24, 1.

Houssay, B.A. (1952). Schweiz. med. Wschr. 39, 57.

Houssay, B.A. (1954). Acta. Physiol. latinoam. 4, 1.

Houssay, B.A. and Galli-Mainini, C. (1950). An. Fac. Med. Montevideo. 35, 5.

Houssay, B.A. and Giusti, L. (1929). C.R. Soc. Biol., Paris. 101, 935.

Houssay, B.A. and Giusti, L. (1930). C.R. Soc. Biol., Paris. 104, 1030.

Houssay, B.A., Giusti, L. and Lascano Gonzalez, J.M. (1929). C.R. Soc. Biol., Paris. 102, 864.

Houssay, B.A. and Lascano Gonzalez, J.M. (1929). C.R. Soc. Biol., Paris. 101, 938.

Houssay, B.A., Penhos, J.C. and Burgos, M.H. (1951). Rev. Soc. argent. Biol. 29, 108.

Humphrey, R.R. (1925). Anat. Rec. 29, 362.

Iwasawa, H. and Asai, O. (1959). J. Fac. Sci. Niigata Univ. 2, 213.

Jenkin, P.M. (1962). Animal Hormones. Pergamon.

Jørgensen, C.B. (1963). Gen. comp. Endocr. 3, 709. (Abstract).

Kambara, S. (1956a). Annot. zool. Jap. 29, 86.

Kambara, S. (1956b). Annot. zool. Jap. 29, 146.

Kambara, S. (1962). Zool. Mag., Tokyo. 71, 328.

Kambara, S. (1963a). J. Fac. Sci. Tokyo Univ. Sect. 4. 10, 189.

Kambara, S. (1963b). Proc. Jap. Acad. 39, 600.

Kambara, S. (1964). Proc. Jap. Acad. 40, 536.

Katzman, P.A. and Doisy, E.A. (1932). J. biol. Chem. 98, 739.

Kehl, R. (1944). Rev. canad. Biol. 3, 131.

Kerr, T. (1965). Gen. comp. Endocr. 5, 232.

Kinoshita, Y. (1933). J. Hiroshima Univ. Ser. B. Div. 1., p. 21.

Landgrebe, F.W. (1939). J. exp. Biol. 16, 89.

Landgrebe, F.W. (1948). Proc. roy. Soc. Edinb. B. 63, 213.

Landgrebe, F.W. and Munday, K.A. (1954). Quart. J. exp. Physiol. 38, 17.

Landgrebe, F.W., Hobson, B.M. and Mitchell, G.M. (1954). Quart. J. exp. Physiol. 39, 309.

Langan, W.B. (1941). Proc. Soc. exp. Biol., N.Y. 47, 59.

Lazo-Wasen, E.A., Neher, G.M., Shoger, R.L. and Zarrow, M.X. (1954). Endokrinologie. 31, 166.

Leslie, J.M. (1890). Proc. zool. Soc. Lond. p. 69.

Levenstein, I. and Charipper, H.A. (1939). Anat. Rec. 75, 123. (Suppl.)

Lodge, P.D.B. and Smith, C.L. (1960). Nature. Lond. 185, 774.

Lofts, B. (1961). Gen. comp. Endocr. 1, 179.

Lofts, B. (1964). Gen. comp. Endocr. 4, 550.

Lofts, B. and Boswell, C. (1960). Nature. Lond. 187, 708.

Lisboa, B.P. and Breuer, H. (1966). Gen. comp. Endocr. 6, 114.

Marshall, F.H.A. (1936). Phil. Trans. B. 226, 423.

Marshall, F.H.A. (1942). Biol. Rev. 17, 68.

McPhail, M.K. and Wilbur, K.M. (1943). J. Pharmacol. 78, 304.

Mintz, B. (1948). Proc. Soc. exp. Biol., N.Y. 69, 358.

Mintz, B. and Witschi, E. (1946). Anat. Rec. 96, 30.

Moore, C.R. and Price, D. (1932). Amer. J. Anat. 50, 13.

Moszkowska, A. (1932). Bull. Biol. 105, 503.

Noble, G.K. (1929). Am. Mus. Novit. 362, 1.

Noble, G.K. and Davis, L.R. (1928). Anat. Rec. 38, 24.

Novelli, A. (1932). C.R. Soc. Biol., Paris. 111, 476.

Nussbaum, N. (1905). Engebn. Anat. EntwGesch. 15, 39.

van Oordt, G.T. (1962). Gen. comp. Endocr. 2, 586.

van Oordt, G.T., Beenakkers, A.M.Th., van Oordt, P.G.W.J. and Stadhouders, A.M. (1954). Acta. Endocr., Copenhagen. 17, 294.

van Oordt, G.T. and Klomp, H. (1946). Proc. K. Ned. Akad. Wet. 49, 565.

van Oordt, G.T. and van Oordt, P.G.W.J. (1955). Mem. soc. Endocr. 55, 25.

van Oordt, P.G.W.J. (1956). Acta. Endocr., Copenhagen. 23, 251.

van Oordt, P.G.W.J. (1960). Symp. zool. Soc. Lond. 2, 29.

van Oordt, P.G.W.J. (1961). Gen. comp. Endocr. 1, 364.

van Oordt, P.G.W.J. (1963). Acta. Physiol. Pharm. Neérl. 12, 89.

van Oordt, P.G.W.J. (1965). Gen. comp. Endocr. 5, 131.

van Oordt, P.G.W.J. and Basu, S.L. (1960). Acta. Endocr. Copenhagen. 33, 103.

van Oordt, P.G.W.J. and Lofts, B. (1963). J. Endocrin. 27, 137.

van Oordt, P.G.W.J. and Schouter, S.C.M. (1961). J. Reprod. Fert. 2, 61.

van Oordt, P.G.W.J., Sluiter, J.W. and van Oordt, G.J. (1951). Acta. Endocr., Copenhagen. 7, 257.

van Oordt, P.G.W.J., Sluiter, J.W. and van Oordt, G.J. (1952). Acta. Endocr., Copenhagen. 9, 155.

Ozon, R. (1967). Gen. comp. Endocr. 8, 214.

Ozon, R. and Breuer, H. (1966). Gen. comp. Endocr. 6, 295.

Ozon, R., Breuer, H. and Lisboa, B.P. (1964). Gen. comp. Endocr. 4, 577.

Penhos, J.C. and Cardenza, A.T. (1952). C.R. soc. Biol., Paris, 146, 132.

Pesonen, S. and Rapola, J. (1962). Gen. comp. Endocr. 2, 425.

Phillips, J.G. and Chester Jones, I. (1957). J. Endocrin. 16, iii.

Ponse, K. (1923). C.R. Soc. Phys. Hist. Nat. Genève. 40, 150.

Ponse, K. (1941). C.R. Soc. Phys. Hist. Nat. Genève. 58, 211.

Ramaswami, L.S. and Lakshman, A.B. (1958). Nature. Lond. 181, 1210.

Randerath, K. (1963). Thin layer chromatography. Verlay Chemie.

Revici, E. (1961). Research in Physiopathology as Basis of Guided Therapy with Special Application to Cancer. Van Rostand, N.Y.

Rimer, Gladys, E.G. (1931). Trans. roy. Soc. S. Afr. 19, pt. 4. 341.

Robbins, S.L. and Parker, F. Jr. (1952a). J. clin. Endocrin. 12, 354.

Robbins, S.L. and Parker, F. Jr. (1952b). J. clin. Endocrin. 12, 744.

Russell, W.M.G. (1954). Behaviour. 7, 113.

Ryan, F. and Grant, R. (1940). Physiol. Zoöl. 13, 383.

Sandoz (1959). MS 222, The Anaesthetic of Choice in Work with Cold-blooded Animals. Sandoz Pharmaceuticals. New Jersey.

Sapeika, N. (1943). S. Afr. J. med. Sci. 8, 115.

Savage, H.M. (1935). Proc. zool. Soc. Lond. pt. iii, 605.

Schrader, M.E.G. (1887). Pflüg. Arch. ges. Physiol. 41, 75.

Schreiber, S.S. and Rugh, R. (1945). J. exp. Zool. 99, 93.

van der Schyff, V. and Zwarenstein, H. (1962). Nature. Lond. 194, 94.

Shapiro, B.G. and Shapiro, H.A. (1934). J. exp. Biol. 11, 73.

Shapiro, H.A. (1936a). J. exp. Biol. 13, 48.

Shapiro, H.A. (1936b). J. exp. Biol. 13, 57.

Shapiro, H.A. (1937). J. exp. Biol. 14, 38.

Shapiro, H.A. (1939). S. Afr. J. med. Sci. 4, (Suppl.) 21.

Shapiro, H.A. (1943). Rev. Soc. argent. Biol. 19, 187.

Shapiro, H.A. and Zwarenstein, H. (1937). J. Physiol. 89, 38P.

Sluiter, J.W., Mighorst, J.C.A. and van Oordt, G.J. (1949). Proc. K. Ned. Akad. Wet. 52, 1214.

Sluiter, J.W., van Oordt, G.J. and Grasveld, M. (1950). Acta. endocr. Copenhagen. 4, 1.

Sluiter, J.W., van Oordt, G.J. and Mighorst, J.C.A. (1950). Quart. J. micr. Sci. 91, 131.

Smith, C.L. (1938). J. exp. Biol. 15, 1.

Smith, C.L. (1955). Mem. Soc. Endocr. 4, 39.

Smith, P.E. (1927). Amer. J. Physiol. 80, 114.

Steinach, E. (1910). Zbl. Physiol. 24, 55.

Sulman, F.G. (1951). Endocrinology. 49, 282.

.

Takahashi, N. (1923). Endocrinology. 7, 302.

Thorburg, J.V. and Hansen, K.K. (1951). Acta. Endocr., Copenhagen, 6, 51.

Tuchmann-Duplessis, H. (1945). Actualities sci. industr. No. 987. Paris.

Waring, H., Landgrebe, F.W. and Neill, R.M. (1941). J. exp. Biol. 18, 11.

Weisman, A.I. and Coates, C.W. (1944). J. Clin. Endocrin. 4, 35.

Welti, E. (1925). C.R. Soc. Biol., Paris. 93, 1490.

Welti, E. (1928). Rev. suisse. Zool. 35, 75.

Wille, (1956). Ann. Endocr., Paris. 17, 326.

Wills, I.A., Riley, G.M. and Stubbs, E.M. (1933). Proc. Soc. exp. Biol., N.Y. 30, 784.

Witschi, E. and Chang, C.Y. (1958). in Comparative Endocrinology. Ed. A. Gorbman. Chapman Hall.

Wolf, O.M. (1928). Anat. Rec. 41, 41.

Wolf, O.M. (1929). Proc. Soc. exp. Biol., N.Y. 26, 692.

Wolf, O.M. (1939). Anat. Rec. 75, (Suppl.) 55.

Worenzowa, M.A. and Blacher, L.J. (1930). Arch. EntwMech. Org. 121, 327. Quoted Dodd. 1960.

Wright, P.A. (1945). J. exp. Zool. 100, 565.

Wright, P.A. (1949). Anat. Rec. 105, 560.

Wright, P.A. (1961a). Gen. comp. Endocr. 1, 20.

Wright, P.A. (1961b). Gen. comp. Endocr. 1, 381.

Wright, P.A. and Flathers, A.R. (1961). Proc. Soc. exp. Biol., N.Y. 106, 346.

Wright, P.A. and Hisaw, F.L. (1946). Endocrinology. 39, 247.

Ying-tien, W. (1963). Acta. Biol. exp. sin. 8, 3.

Zahl, P.A. (1937). Z. Mikr.-anat. Forsch. 41, 303.

Zondek, B. and Ascheim, S. (1927). Arch. Gynäkol. 130, 1.

Zwarenstein, H. (1936). Proc. roy. Soc. S. Afr. Oct. 21.

Zwarenstein, H. (1937). Nature. Lond. 139, 112.

Zwarenstein, H. (1939). S. Afr. J. med. Sci. 4, (Suppl.) 18.

Zwarenstein, H. (1942). Trans. roy. Soc. S. Afr. 22, xii.

Zwarenstein, H. and Schrire, I. (1932). Proc. roy. Soc., Edinb. 52, 323.

Zuber-Vogelli (1952). Arch. Anat., Strasbourg. 35, 77.

The abbreviated titles used in this bibliography are those recommended in the World List of Scientific Periodicals.

APPENDIX.

Paper published jointly with

B.M. Hobson entitled:

The anaesthetic action of di-ethyl ether,

ethyl carbamate and tricaine

methanesulfonate upon Xenopus laevis.

Reprinted from
SMALL ANIMAL ANAESTHESIA
Proceedings of a Symposium held in London, July, 1963
PERGAMON PRESS
OXFORD . LONDON . EDINBURGH . NEW YORK . PARIS . FRANKFURT
1964

THE ANAESTHETIC ACTION OF DI-ETHYL ETHER, ETHYL CARBAMATE AND TRICAINE METHANESULFONATE UPON *XENOPUS LAEVIS*

B. M. HOBSON AND B. G. TOWNSEND
University of Edinburgh

INTRODUCTION

Ethyl ether and ethyl carbamate (urethane) are commonly used to anaesthetize laboratory animals. Ether in high concentrations acts rapidly but causes the animal to become stressed. Furthermore the inflammability of ether precludes its use when a cautery is necessary.

Aquatic vertebrates may be anaesthetized with urethane either by immersion in an aqueous solution or by injection. Although urethane has a more moderate action than ether upon the organism, attention has been drawn to its carcinogenic properties (Ball and Cowen, 1959; Revici, 1961).

Recent experience with tricaine methanesulfonate (M.S. 222 Sandoz) has suggested that it might be the ideal anaesthetic for poikilothermic vertebrates. Among the Amphibia, both larval and adult Urodela and Anura have been successfully anaesthetized with tricaine (Sandoz, 1959). However, detailed information about the response of individuals when injected with or immersed in solutions of tricaine is available for only a few species.

We have compared the suitability of tricaine for anaesthetizing *Xenopus laevis* with that of ether and urethane. The effect of temperature, repeated use, and body weight upon the time taken for anaesthesia and recovery has been investigated. Certain other aspects of these anaesthetics, such as their effect upon blood sugar level, colour change, and the release of spermatozoa have been examined.

MATERIAL AND METHODS

Male and female *Xenopus* adults, of approximately the same weight and in groups of 10, were anaesthetized by two methods. The toads were either immersed in an aqueous solution of the anaesthetic, prepared immediately before use, or the solution was injected into the dorsal lymph sac (D.L.S.). Preliminary trials indicated that the following concentrations would be suitable for immersion anaesthesia; tricaine 0·13 per cent, urethane 2·5

per cent and ether 4·0 per cent. 0·01 ml of a 1·3 per cent solution of tricaine in distilled water was injected with a tuberculin syringe per gram body weight. The dose necessary to produce anaesthesia with urethane was found to be most closely related to body weight by the formula $x = 3(y + 8)$ where x was the dose in milligrams and y was the weight of the toad in grams. Solutions of urethane were neutralized before injection. Ether was not injected.

Toads were considered to be anaesthetized if no foot retraction reflex occurred when the digits of the hind legs were pinched firmly three times with a pair of dissecting forceps. Two stages of recovery were observed; "partial", when the withdrawal reflex reappeared, and "complete", when toads placed under water, ventral surface uppermost, righted themselves three times in succession and exhibited normal swimming movements.

Rapid post-operative recovery is advantageous and the time taken by toads to recover in water and in air after being anaesthetized was compared. Partial recovery of half of each group took place in running water. The toads were supported upon a grid and immersed except for their nostrils. The other half of each group began their recovery in air, being laid upon damp cotton wool in an open dish until partial recovery was attained. They were then placed on the grid in running water until completely recovered. Toads were not returned to the aquarium until they were capable of swimming normally.

To standardize, as far as possible, the conditions under which the experiments were carried out, the water in which the toads were kept for at least an hour before each experiment, the anaesthetic solutions used for immersion, and the water in which the toads were allowed to recover, were maintained at 22° ± 1°C. During partial recovery in air, no attempt was made to accurately control the ambient temperature which fluctuated between 20° and 24°C.

During the experiment in which temperature was the controlled variable, all the toads and containers of anaesthetic solutions were kept in a tank of water maintained at the required temperature to ±0·5°C by a thermostatically controlled water heater and circulator. Twenty-four hours later the toads were anaesthetized, and, for the duration of the experiment, were kept in beakers of anaesthetic solution in the temperature regulated tank.

Blood sugar levels were estimated by the method of Landgrebe and Munday (1954). The toads were pithed, the heart exposed and the pericardium removed. The ventricle was allowed to hang through a hole in a filter paper, the apex cut and the blood collected in a dry crucible. In this way blood was obtained quickly and without contamination with other body fluids. Blood was taken from the crucible with an accurate 0·1 ml pipette. Two or three 0·1 ml samples were usually obtained from each animal. The blood sugar was estimated and the mean value in milligrams

per cent recorded. Previous experience had shown that the variation in blood sugar levels was considerably less in "starved" animals than those recently fed. For this reason, toads used for blood sugar estimations were kept without food for 2 weeks before being anaesthetized.

Melanophores of *Xenopus*, after being anaesthetized with ether, tricaine and urethane, were examined microscopically. The degree of expansion or contraction of the melanophores was recorded in terms of the arbitrary melanophore index (m.i.) of Hogben and Gordon (1930). Unity stands for the fully contracted state and 5 the value assigned to maximum expansion.

Male *Xenopus* immersed in a mixture of 1 vol of ether to 9 vol of water, will spermiate. The effect of ether, tricaine and urethane upon the release of spermatozoa was investigated. Four and 24 hr after anaesthesia the urine of the male *Xenopus* was examined for the presence of spermatozoa using the method of Hobson and Landgrebe (1954).

RESULTS

Anaesthesia by Immersion

The times taken for male and female toads to become anaesthetized, and to recover partially and completely after ether, were not affected by the weight of the animal. The figures obtained suggest that partial and complete recovery of the male toad is delayed compared with that of the female after ether treatment. When tricaine and urethane were used, the time taken by toads to become anaesthetized and recover lengthened as their weight increased. Following the use of these two anaesthetics the female toad in general recovered more rapidly than the male. The times taken for anaesthetization and recovery were shortest with ether. There was little difference between the time taken to anaesthetize toads with tricaine compared with urethane, but toads took longer to recover completely after urethane anaesthesia (Table 1).

It was found that toads immersed in solutions of tricaine or urethane revived more rapidly when recovery took place in water. The time taken to recover completely was increased by 30 per cent when partial recovery occurred in air. Toads recovered equally quickly after ether anaesthesia, whether this took place in air or water.

Repeated Anaesthesia

The tolerance of *Xenopus* to repeated anaesthesia was tested in the following way. Ten male and 10 female toads, each about 50 g in weight, were anaesthetized on 6 successive days with tricaine. Four weeks later the same animals were anaesthetized daily for 6 days with urethane and after a further 4 weeks were anaesthetized on 6 successive days with ether.

The time taken by these 20 toads to become anaesthetized and to recover

TABLE 1. TIME TAKEN FOR GROUPS, 10 TOADS PER GROUP, OF MALE AND FEMALE XENOPUS TO BECOME ANAESTHETIZED AND RECOVER AFTER IMMERSION IN SOLUTIONS OF ETHER, TRICAINE AND URETHANE

Xenopus		4·0 per cent Ether			0·13 per cent Tricaine (MS 222)			2·5 per cent Urethane		
		Time (in minutes) taken to attain			Time (in minutes) taken to attain			Time (in minutes) taken to attain		
Sex	Group mean weight g	Anaesthesia	Recovery		Anaesthesia	Recovery		Anaesthesia	Recovery	
			Partial	Complete		Partial	Complete		Partial	Complete
Male	23·6±2·3*	2·5±0·9	19·9± 7·3	31·6± 5·9	5·4±2·1	15·1± 6·5	25·1±11·3	8·5±1·9	22·1± 7·0	41·3±15·4
	34·8±2·1	2·8±0·6	23·8± 9·2	31·0± 6·9	7·3±2·7	21·4± 1·2	28·5± 8·1	10·7±2·5	21·0± 7·5	50·0±20·8
	50·6±2·5	3·6±0·8	19·8± 7·7	38·5± 4·7	14·7±5·7	40·5±12·2	59·1±25·3	14·2±2·7	41·6±17·2	91·6±20·4
	64·0±2·7	4·0±0·7	18·9± 5·2	37·3± 7·8	16·8±6·8	45·7±11·5	69·5±18·0	17·9±3·9	43·3±17·3	90·3±18·1
Female	23·8±2·3	2·0±0·6	11·4± 4·8	18·5± 3·8	6·0±2·2	21·2±10·8	29·2±10·9	7·0±1·9	13·5± 4·8	31·9± 9·5
	34·1±1·9	3·6±1·5	18·8± 5·1	32·8± 2·5	10·0±3·1	20·6± 3·7	31·4± 2·8	11·6±1·8	24·6± 8·6	53·9±15·8
	52·7±2·7	3·7±0·6	14·9±11·5	28·3± 6·9	14·6±4·4	27·8±10·9	40·0±16·4	13·1±2·6	35·0±11·5	67·2±20·2
	64·4±3·0	3·2±0·5	16·1± 2·4	30·1± 3·8	15·0±3·9	27·3± 9·0	32·9±10·6	16·8±3·1	40·0±13·4	65·6±11·6
	78·5±2·8	3·7±0·9	14·2± 2·0	31·9± 9·4	16·8±6·5	25·9± 6·5	31·8± 8·1	18·9±2·3	41·5± 9·8	80·3±18·6
	92·2±3·7	3·5±0·8	16·6± 6·9	31·8±10·6	20·2±5·9	22·8± 6·8	34·8±11·6	17·6±4·7	36·3±13·8	80·1±30·0

*Each figure is followed by the standard deviation of the mean.

TABLE 2. EFFECT OF TEMPERATURE UPON THE TIME, IN MINUTES, TAKEN BY GROUPS, 3 MALE AND 3 FEMALE TOADS PER GROUP, OF XENOPUS TO BECOME ANAESTHETIZED AND RECOVER

	Temperature of anaesthetic solutions								
	16°C			21°C			26°C		
		Recovery			Recovery			Recovery	
Anaesthetic	Anaesthesia	Partial	Complete	Anaesthesia	Partial	Complete	Anaesthesia	Partial	Complete
Ether 4 per cent	5·0±1·4*	17·3± 5·9	28·6± 9·8	4·2±1·4	13·1±5·3	26·7±9·8	3·9±0·4	12·8±3·3	26·1±5·5
Tricaine 0·13 per cent	14·9±4·9	24·1± 6·9	44·3±10·5	11·2±2·4	17·0±2·4	26·8±7·0	9·8±2·8	15·5±2·5	24·2±4·5
Urethane 2·5 per cent	19·7±5·7	29·3±10·1	107·2± 8·2	19·6±3·2	30·7±8·2	75·3±5·9	13·1±2·5	22·5±3·2	53·9±8·8

*Each figure is followed by the standard deviation of the mean.

was not altered by this treatment. In the order in which they were used, one anaesthetic did not influence the response of the toads to the next anaesthetic.

Effect of Temperature

The effect of temperature on the speed and duration of anaesthesia was investigated. Three groups of toads, each containing 3 males and 3 females of similar weight, were anaesthetized by immersion in 4 per cent ether, 0·13 per cent tricaine and 2·5 per cent urethane. One group was used for each anaesthetic. During the first experiment the temperature of the anaesthetic solutions and water in which the toads recovered was kept at 16°C. During the second and third experiments the temperature of all solutions was kept at 21°C and 26°C respectively. Anaesthesia and subsequent recovery occurred more rapidly as the temperature increased (Table 2).

Anaesthesia by Injection

Forty male *Xenopus*, weighing between 23 g and 62 g, and 60 females, between 24 g and 87 g, were injected with a dose of urethane calculated to produce complete anaesthesia and all but 7 toads responded. The reaction of both male and female *Xenopus* to urethane was unpredictable and the time which elapsed between injection and anaesthesia varied from 3 to 24 min. Partial recovery in air took between 28 and 84 min and complete recovery between 67 and 126 min from the time of injection.

TABLE 3. TIME TAKEN FOR GROUPS, 10 TOADS PER GROUP, OF MALE AND FEMALE XENOPUS TO BECOME ANAESTHETIZED AND RECOVER AFTER AN INJECTION OF 130 μG OF TRICAINE PER GRAM BODY WEIGHT

Xenopus		Time, minutes, taken to attain		
Sex	Group mean weight g	Anaesthesia	Recovery	
			Partial	Complete
Male	23·1±2·4*	3·7±1·2	23·9± 6·7	37·1± 8·4
	34·5±0·6	3·8±1·5	18·9± 7·1	33·5± 8·4
	50·8±1·5	5·1±3·0	19·3± 6·5	31·2± 4·9
	62·0±2·7	4·4±1·8	18·3± 5·5	27·0± 8·1
Female	22·9±1·5	3·2±0·9	18·8± 5·1	29·2± 5·4
	34·5±1·0	5·7±2·5	20·2± 5·2	35·2± 7·2
	50·2±2·4	3·4±1·2	23·6± 6·0	37·2± 5·4
	61·9±3·3	3·4±0·7	19·5±12·8	29·6±10·9
	77·1±3·0	4·6±1·6	18·6± 7·4	30·3± 8·5
	94·8±3·5	4·2±1·5	20·7± 6·2	32·4± 7·9

*Each figure is followed by the standard deviation of the mean.

One hundred *Xenopus* were injected with 130 μg of tricaine per gram body weight. Two toads only were not anaesthetized by this dose. The times taken by the groups of male and female *Xenopus* of varying weight to become anaesthetized, to recover partially and completely are shown in Table 3.

Male and female *Xenopus* respond similarly to injected tricaine.

Anaesthesia of Xenopus Larvae

Ten tadpoles, between 30 mm and 50 mm in length, were anaesthetized as a group by immersion in aqueous solutions of tricaine. Solutions of the following concentrations were used: 1 in 1000; 1 in 5000; 1 in 10,000; and 1 in 15,000; and the times taken for the group to become anaesthetized were 0·5 min, 1 min and 6 min respectively. Tadpoles were considered to be anaesthetized when all tail movements had ceased and the animals dropped to the bottom of the container. After anaesthesia, the tadpoles were returned to the aquarium and allowed to recover. The time taken for this was 18 min after anaesthetization in the 1 in 1000 solution, and between 3 min and 3·5 min when the lower concentrations were used.

Miscellaneous Effects of the Anaesthetics

Stress. A measure of the metabolic disturbance, due to immersion in ether, or tricaine or urethane, was obtained by estimating the glucose content of the toads' blood at various times after anaesthesia. The blood sugar level of uninjected controls and controls injected with distilled water was also measured. These results were compared with the hyperglycaemia produced in *Xenopus* after an injection of 5 μg of adrenaline hydrochloride (Table 4).

The hyperglycaemic action of ether, urethane and to a lesser extent tricaine was similar to that following the injection of adrenaline.

Spermiation. In the concentration used (Table 1), none of the anaesthetics had any gametokinetic activity. It was found that a 5 per cent solution of urethane caused the release of spermatozoa in 1 of the 10 toads in the experiment. Eight per cent ether and 0·26 per cent tricaine did not cause spermiation.

Colour change. Male *Xenopus* were adapted to a white background so that the melanophores were contracted (m.i. 1·0), and were anaesthetized in groups of 12 animals. Toads were immersed in solutions of 4 per cent and 8 per cent ether, 0·13 per cent and 0·26 per cent tricaine, and 2·5 per cent and 5 per cent urethane. Ten toads each weighing 38 g were injected with tricaine and 10 toads of similar weight were injected with urethane. Anaesthetics, whether administered by immersion or injection, did not cause melanophore expansion in any of the toads. Twenty white-adapted animals were each injected with 10 μg of adrenaline hydrochloride.

TABLE 4. BLOOD SUGAR LEVELS OF XENOPUS AT VARIOUS TIMES AFTER ANAESTHESIA WITH ETHER, TRICAINE, URETHANE AND FOLLOWING AN INJECTION OF 5 μG ADRENALINE HYDROCHLORIDE AND DISTILLED WATER

Treatment	Number of		Blood sugar mg per cent			
	Toads	Estimations	20 min	60 min	120 min	240 min
Immersion 4 per cent ether	11	25	54·4	104·3	117·1	118·7
Immersion 0·13 per cent tricaine	11	26	52·5	73·1	91·6	81·5
Immersion 2·5 per cent urethane	10	19	45·0	74·3	80·7	113·6
Injected 5 μg adrenaline hydrochloride	8	19	64·6	70·7	102·0	126·0
Injected control	4	8				37·5
Uninjected control	6	17	31 mg per cent			

TABLE 5. EFFECT OF ETHER, TRICAINE, URETHANE AND ADRENALINE UPON MELANOPHORES OF MALE XENOPUS ADAPTED TO A BLACK BACKGROUND

Number of toads	Treatment	Mean m.i.		Time, minutes, for melanophores to	
		Before treatment	After treatment	reach maximum contraction	return to pre-treatment state
12	Immersed, 4 per cent ether	5·0	3·1	48	264
12	Immersed, 0·13 per cent tricaine	4·8	2·7	10	120
10	Injected, 4·94 mg tricaine	4·8	2·7	15	280
12	Immersed, 2·5 per cent urethane	4·9	4·2	12	85
10	Injected, 138·0 mg urethane	4·8	2·8	90	285
10	Injected, 10 μg adrenaline hydrochloride	5·0	3·0	45	200

Melanophores of some of the toads receiving adrenaline expanded and the maximum response of the group, m.i. 1·6, occurred 2 hr after injection. A similar experiment was performed with male *Xenopus* adapted to a black background so that the melanophores were expanded (m.i. 5·0). Ether, tricaine, urethane and adrenaline hydrochloride caused the melanophores of all toads to contract (Table 5).

Mucification. When *Xenopus* were imersed in ether water mixtures they secreted large quantities of mucus from their cutanuos glands. Immersion in, or injection of, either tricaine or urethane did not cause this secretion.

DISCUSSION

In *Xenopus laevis* anaesthesia is most quickly induced by immersion in an ether water mixture, and recovery is also the most rapid. This rapid action of ether is the only advantage it possesses over tricaine and urethane. Ether causes severe mucification which makes the toads unpleasant to handle. There is a risk of fire when a cautery is used, and the effects of ether vapour on the operator are unpleasant. The level of stress induced by ether, tricaine or urethane is reflected by the degree of hyperglycaemia produced. Anaesthetization by ether, tricaine or urethane induces a hyperglycaemia comparable with that caused by the injection of 5 μg of adrenaline hydrochloride. Urethane had distinct disadvantages; recovery is delayed after anaesthesia produced by immersion, and when given by injection the response of the toads is unpredictable. Furthermore, there is evidence that urethane induces tumours in rats and mice, and may be carcinogenic in man (Ball and Cowen, 1959; Revici, 1961). After immersion in a solution of tricaine, anaesthesia occurs in a conveniently short time, except when heavy toads are used. Anaesthesia is induced within 3–6 min by injecting a dose of tricaine related to the weight of the toad. Recovery from the anaesthetic effects of tricaine occurs quickly whichever method is used. In other respects there is little difference between the actions of the three anaesthetics. In the doses usually employed no gametokinetic activity is observed. Doubling the concentration causes spermiation when urethane is used, but ether and tricaine have no effect. However, when "free" ether is present, in an ether–water mixture, spermiation is induced in male *Xenopus*.

Immersion of "white-adapted" toads in any of the three anaesthetics does not cause melanophore expansion. The injection of 10 μg of adrenaline hydrochloride into 20 toads causes slight melanophore expansion (m.i. = 1·6) in some, but not all toads. This degree of response is similar to that obtained by Graham (1959) who found that 20 μg adrenaline produced a small expansion of the melanophores of *Xenopus* adapted to a white background. More recently Landgrebe and Waring (1962) have stated "that

doses up to 100 μg of adrenaline have little or no effect on melanophore expansion". Toads adapted to a black background exhibit melanophore contraction and become pale when immersed in, or are injected with, tricaine, ether or urethane. This melanophore-contracting effect of the anaesthetics is comparable with that produced by an injection of 10 μg of adrenaline.

The results obtained in the investigation suggests that tricaine is a more useful anaesthetic for *Xenopus laevis* than urethane or ether.

SUMMARY

1. The anaesthetic effect of ether, tricaine or urethane was studied on male and female toads (*Xenopus laevis*).
2. When toads were immersed in solutions of these anaesthetics, the time taken for anaesthesia, and for partial and complete recovery to occur, was most rapid after ether.
3. The tolerance of toads to ether, tricaine or urethane was unaltered by repeated anaesthesia.
4. Anaesthesia and subsequent recovery occurred more rapidly as the temperature of the anaesthetic solutions, and the water in which the toads recovered, was increased.
5. The behaviour of toads anaesthetized by an injection of urethane was uncertain. Toads injected with tricaine behaved in a predictable manner and recovery was rapid. Ether was not injected.
6. *Xenopus* tadpoles were anaesthetized with tricaine and the time taken for anaesthesia and recovery was related to the concentration of tricaine used.
7. The blood sugar level of toads anaesthetized by ether, tricaine or urethane was comparable with that produced by an injection of 5 μg of adrenaline hydrochloride.
8. Spermiation occurred if a 5 per cent solution of urethane was used or when "free" ether was present in the ether–water mixture.
9. Melanophores of *Xenopus* adapted to a white background did not expand when the toads were immersed in solution of ether, tricaine or urethane. The melanophores of toads adapted to a black background contracted when the animals were immersed in any of the three anaesthetics.
10. Intense mucification of the toads' skin occurred after ether anaesthesia but not after using tricaine or urethane.
11. Tricaine is considered to be superior to ether or urethane as an anaesthetic for *Xenopus laevis*.

ACKNOWLEDGEMENT

The authors wish to thank Dr. H. Holgate of Sandoz Ltd., for a generous supply of tricaine methanesulfonate.

REFERENCES

J. N. BALL and P. N. COWEN (1959). Urethane as a carcinogen and as an anaesthetic for fishes. *Nature, Lond.*, **184,** 370.

J. D. P. GRAHAM (1959). Antagonism by 2-haloalkylamine compounds of some actions of adrenaline noradrenaline and isoprenaline on *Xenopus laevis*. *Arch. Int. Pharmacodyn.* **181,** 317–326.

B. M. HOBSON and F. W. LANDGREBE (1954). Biological assay of pituitary, serum and chorionic gonadotrophins and of adrenaline using male *Xenopus laevis*. *Quart. J. Exp. Physiol.* **39,** 23–27.

L. HOGBEN and C. GORDON (1930). The separate identity of the pressor and melanophore principles. *J. Exp. Biol.* **7,** 286–292.

F. W. LANDGREBE and K. A. MUNDAY (1954). A modified Folin–Malmros technique for the estimation of blood sugar, particularly suitable for low values or for micro work. *Quart. J. Exp. Physiol.* **38,** 17–22.

F. W. LANDGREBE and H. WARING (1962). Melanophore expanding activity. In *Methods in Hormone Research*, Ed. R. J. Dorfman, Vol. 2, Academic Press, New York.

SANDOZ (1959). MS 222 Sandoz, *The Anaesthetic of Choice in Work with Cold-blooded Animals*. Sandoz Pharmaceuticals, New Jersey.

E. REVICI (1961). *Research in Physiopathology as Basis of Guided Therapy with Special Application to Cancer*. Van Nostrand, New York.

Printed in Great Britain by Charles Birchall and Sons Limited, Liverpool and London